MOLECULAR TAXONOMY
of
Paracoccus halodenitrificans
Aeromonas salmonicida
and
Enterococcus seriolicida

by

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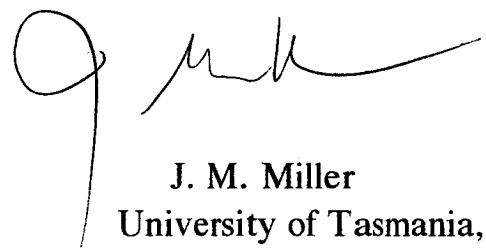

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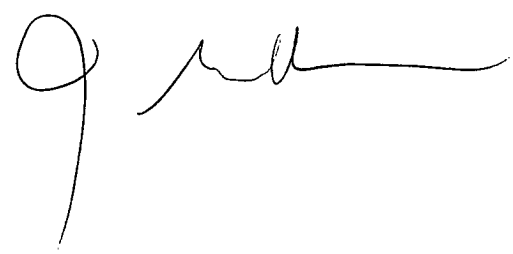

LIST OF ABBREVIATIONS

Genera

A.	*Aeromonas*
B.	*Bacillus*
D.	*Deleya*
E.	*Escherichia*
Ent.	*Enterococcus*
H.	*Halomonas*
Hv.	*Halovibrio*
L.	*Lactococcus*
O.	*Oceanospirillum*
On.	*Onchorhynchus*
P.	*Paracoccus*
Ps.	*Peudomonas*
Rsp.	*Rhodopseudomonas*

Culture Collections

ACAM	Australian Collection of Antarctic Microorganisms, Univerity of Tasmania
ATCC	American Type Culture Collection, Rockville, Maryland, USA
CCM	Czechslovak Collection of Microrganisms, Brno, Czechoslovakia
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany
LMG	Laboratorium Microbiologie Rijksuniversiteit Gent, Gent, Belgium
NCDO	National Collection of Dairy Organisms, Reading, UK
NCMB (NCIMB)	National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland

Data Bases

GSDB	Genome Sequence Data Base (formerly Genbank)
EMBL	European Molecular Biology Laboratory
RDB	Ribosomal Database

Other Abbreviations

A	adenine
ANGIS	Australian National Genomic Information Service
ATP	adenosine triphosphate
C	cytosine
CE	carp erythrodermatitis
dH_2O	distilled water
ddH_2O	distilled, deioinised water
DPIF	Dept. Primary Industry and Fisheries
DMK	dimethylmenaquinone
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotriphosphates
EDTA	di-sodium ethylenediaminetetraacetate
G	guanine
GUD	goldfish ulcer disease
HPLC	high performance liquid chromatography
MK	menaquinone
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PYRA	pyrrolidonylarylamidase
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAse	ribonucleic acid nuclease
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecylsulphate
sp./spp.	species (singular/plural)
T	thymine
UV	ultraviolet

ABSTRACT

The sequence of the 16S rRNA molecule has become accepted as a systematic fingerprint allowing the evolutionary history of an organism and its phylogenetic status at various taxonomic levels to be characterised. Collection of sequence data and their comparison under explicitly defined and widely, though tentatively, accepted algorithms provides much insight into the relationships between organisms.

In the study of microbiology, classical taxonomy has been hindered by a paucity of morphological distinction between bacteria. With the acceptance of molecular systematics, many bacteria and groups of bacteria are now being reorganised into a system that reflects both their histories and their relationships, and is consequently more stable than heretofore.

This thesis deals with the classification of three bacteria on the basis of their 16S rRNA sequences.

* *Paracoccus halodenitrificans*
Various chemotaxonomic and molecular data suggest that this species is generically misplaced. 16S rDNA sequence data place the type species, *P. denitrificans*, in the a-subclass of the Proteobacteria. 16S rDNA sequence analysis undertaken in this work places *P. halodenitrificans* within the family Halomonadaceae in the g-subclass of the Proteobacteria.

* *Enterococcus seriolicida*
A bacterial strain isolated from a Tasmanian salmon farm bears strong resemblance to *E. seriolicida* (ATCC 49156^T = YT-3). In 1993, workers in Spain suggested that *E. seriolicida* and *Lactococcus garvieae* are synonymous by 16S rRNA sequence identity, though no sequence data for the former was published. Analysis of the 16S rRNA sequence of *E. seriolicida* in this work assigns the species to the genus *Lactococcus*. The sequence of *E. seriolicida* differs from the published 16S rRNA sequence of *L. garvieae* in only seven positions. These differences and their significance are discussed.

* *Aeromonas salmonicida*
A bacterial strain isolated on a northern Tasmanian fish farm from a skin lesion of the greenback flounder *Rhombosolea tapirina* was presumed an endemic atypical subspecies of the salmonid pathogen *Aeromonas salmonicida.* Clarification is necessary for an accurate assessment of its significance for the fishing industry. 16S rRNA sequence of this organism shows 100% identity with that of the species *Aeromonas salmonicida* subsp. *masoucida* and *achromogenes*, despite phenotypic differences between the bacteria. The genus *Aeromonas* has an unusually high degree of sequence similarity among the 16S rRNA genes of its members. This may make taxonomic clarification by this criterion dubious.

The definition of a species on the basis of the sequence of its 16S rRNA molecule has ramifications beyond taxonomy. Synthetic oligonucleotide probes designed to complement specifically unique regions the 16S rRNA or rDNA can rapidly and accurately identify organisms for many purposes. The salmonid industry in Tasmania has been free of the major diseases responsible for devastating losses in overseas fisheries. However, two pathogenic bacteria have been isolated locally, *Enterococcus seriolicida* and a presumed endemic subspecies of *Aeromonas salmonicida.* Molecular probes directed against the rDNA of these organisms are required that will allow them to be identified rapidly, their occurrence investigated and their epidemiology traced efficiently should the need arise. A simple diagnostic assay, compatible with normal pathological laboratory routine, is necessary. Therefore these probes have been designed for use as primers in a PCR-based assay.

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1. MOLECULES IN BACTERIAL SYSTEMATICS

1.1. Introduction

For over a century, the classification of bacteria has been fundamentally a process of description and diagnosis based on phenotype. However, as the phylogenetic basis of metazoan taxonomy was recognised and developed, a comparable "natural" classification system based on underlying historical relatedness of bacteria became an important objective of microbiology. The data available to the microbial taxonomist were, however, far less informative than those available to the taxonomist of multicellular organisms. The phenotypes of the metazoans are complex, diverse and amenable to distinction on many levels (gross morphology, embryogenesis, biochemistry, genetics, fossil history etc.), and these distinctions parallel, at least approximately, the evolutionary relationships between organisms. The relatively simple phenotypes of bacteria, on the other hand, offer very little by which to trace their evolutionary relationships. Such is the paucity of phylogenetically useful indicators among bacterial phenotypes that, by the middle of this century, it was generally accepted that attempts to construct a phylogenetic history of the protists were "doomed to failure" through lack of "criteria of truly phylogenetic significance" (van Niel, 1955).

An organism is the product of its genetic substance. This substance occurs as sequences of nucleic acids, and derivatively in the sequences of proteins which are encoded by nucleic acids. These biological macromolecules contain information that reflects not only their function but also their evolutionary history (Zuckerkandl & Pauling, 1965). As with phenotypic characters, phylogenetic deductions can be made on the basis of accrued differences in primary structures of molecular homologues. Insofar as the sequences of nucleic acids and proteins are linear and composed of large numbers of a small, finite set of precise and unambiguous characters, differences can be quantified and analysed statistically. Such quantification allows derivation of the phylogenies of these molecules without the subjectivity intrinsic to phylogenetic inference based on phenotypic differences. If it can be shown that such macromolecules evolved as integral parts of the organisms containing them, then their histories and thus their phylogenetic relations will reflect the organisms' own.

With comparative analysis of primary molecular structures in cells, a phylogeny of the prokaryotes that is stable and internally consistent is now possible.

1.2. Techniques in Molecular Systematics

Several ways of accessing the historical information in biological molecules are available.

* Proteins are compared
- indirectly in immunological studies of homologous antigens
- by electrophoretic separation of cell proteins
- as amino acid sequences of homologous proteins.

* Deoxyribonucleic acids (DNA) are compared
- by base composition of genomes i.e. relative G+C content
- as whole genomes in DNA-DNA hybridisations
- as the nucleotide sequences of genes coding homologous proteins or non-translated transcripts (RNA).

* Ribonucleic acids (RNA) are compared
- in rRNA-DNA hybridisation, matching the rRNA molecules of one organism against the corresponding gene for those molecules in another organism
- as partial sequence data, known as rRNA "catalogues" - oligomeric nuclease digestion products of ribosomal RNA molecules
- as complete ribosomal RNA sequences determined by reverse transcription.

These various techniques all access the information lode of primary biological molecules. However, the information so derived differs in kind. Nucleic acid hybridisation data (DNA-DNA, rRNA-DNA), binary similarity coefficients of rRNA catalogue analysis, and antibody cross-reactivity are fundamentally measures of molecular similarity. They can necessarily only be made between the attributes of two organisms at a time. (16S rRNA catalogues are included in this category because, although the data are based on nucleotide sequences, it is not the sequences themselves that are compared but the occurrence of subsets of them. The "similarity

coefficient" that defines these differences numerically is calculated: $S_{AB} = 2N_{AB} / N_A + N_B$. It is clear that the measure S_{AB} can only ever compare data from the two organisms A and B.) Such measures require pairwise comparisons of all organisms under consideration. Consequently computations become unwieldy over many taxa and the difficulties of reproducing experimental exactitude between laboratories and over time makes broad-ranging comparisons difficult. Data cannot be accumulated and referenced for new organisms. All comparisons must be made *de novo*. Molecular sequence data, on the other hand, comprise a simple linear set of units for each organism which is directly comparable to the sets of all other organisms. Sequence sets can be stored as an ever-increasing database of aligned homologous characters against which sequences of new organisms can be immediately evaluated.

These techniques also render up phylogenetic information that differs in the taxonomic level at which it can be applied. These variations will be addressed under each sub-section.

1.2.1 Proteins

Direct comparison of gene products has not been extensive in microbiological taxonomy, but indirect comparisons have played a significant part in classification.

1.2.2.1. Immunological analysis

Immunological comparisons are an indirect measure of primary structural differences of homologous proteins. There is strong correlation between percentage substitution of amino acids and immunological similarity (Arnheim & Wilson, 1967; Schleifer & Stackebrandt, 1983). Worthy resolution of organisms is achieved only when compared homologues differ in amino acid sequence by less than 30-40%, and when these differences are predominantly at the conformational surface of the protein and therefore accessible to antisera (Prager & Wilson, 1971).

As with direct analyses of amino acid sequences, discerning choice of proteins allows the resolution of bacterial relatedness at widely varying taxonomic levels. Catalase, for instance, can be effectively used for immunologic distinction of closely related strains, in excellent agreement

with DNA-DNA hybridisation results (Rupprecht & Schleifer, 1979). Glutamine synthetase, on the other hand, distinguishes genera that are distantly related (Baumann et al., 1980), in agreement with DNA-rRNA hybridisation results.

1.2.1.2. Electrophoretic analysis

Electrophoretic separation of whole cell proteins, of particular subsets of cellular proteins or of isozymes allows fast and easy comparisons of different organisms. Many strains may be examined simultaneously. Data from cellular protein profiles is amenable to cluster analysis, and can be stored for subsequent access. Results of whole cell protein analyses correlate well with DNA-DNA hybridisation results (Jackman, 1985) and are taxonomically applicable at or below species level (Costas, 1990; Costas et al., 1992).

The major problem with protein comparisons is the formidable effect of growth conditions on constituent cell proteins. The expression of proteins is often inducible under circumstances not fully understood, and reproducibility of growth conditions between strains cannot be indemnified between laboratories and through time (Schleifer & Stackebrandt, 1983).

Polymorphic enzymes (isozymes) such as dehydrogenases and esterases are compared on the basis of their sequence-determined electrophoretic mobility in one-dimensional gels. Organisms can then be grouped by shared polymorphisms. DNA-directed RNA polymerases analysed in this way have proved especially cogent in definition of archeal phylogeny (Stetter et al., 1981).

1.2.1.3. Amino acid sequences

Proteins have been completely sequenced for a relatively limited number of bacterial groups (Fox & Stackebrandt, 1987) and their contribution to bacterial systematics has been correspondingly restricted. This has been partly due to a hesitation in confidently identifying truly orthologous proteins (Sneath, 1988). Protein genes can be duplicated early in evolutionary history, with subsequent independent structural divergence with or without functional divergence (Ambler, 1985). Parallelisms and

convergence within protein sequences are common phenomena (Guise et al., 1982). Mutationally silenced "pseudogenes" are subject to unconstrained genetic drift that makes them useful at only very short genetic distances (Kimura, 1983; Harris et al., 1984). Many silent substitutions occur between functionally equivalent amino acids (Kimura, 1968; Kimura, 1969) Lateral transfer of protein-encoding genes is well-documented (eg. β-lactamase [Kirby et al., 1992], alkaline phosphatase and α-amylase [Smith et al., 1991]) and pernicious to phylogenetic comparison. These factors introduce uncertainty into the identification of historically equivalent molecules and have disinclined the bacterial taxonomist from establishing a dependence on, and concomitantly a database of protein sequences, especially when dependable and recognised homologues are available in the rRNA molecules. Furthermore, many proteins evolve so rapidly that their information content is too low for insights across the vast genealogical distances of bacterial evolution (Ambler, 1985).

Because of the differing rates at which various proteins have evolved, different molecules have been used to establish different branches of bacterial phylogeny, and at different taxonomic levels. With prudent choice of proteins, these phylogenies conform reasonably well with those established by rRNA analysis (Olsen & Woese, 1993). The RNA polymerases (Puhler et al., 1989), proton-translocating ATPases (Gogarten et al., 1989), *nif* nitrogenase genes (Hennecke et al., 1985) and elongation factor G (EF-G) (Cammarano et al., 1992) provide reconstructions essentially the same as 16S rRNA. The c-type cytochromes give a more limited but comparable phylogeny (Ambler et al., 1985; Woese et al., 1980; Meyer et al., 1986). The bacterial ferredoxins also deliver a phylogeny basically congruent with the small subunit rRNAs, after the various intragenic duplications, deletions and rearrangements that characterise the ferredoxin genes have been accommodated (George et al, 1985).

"... the demand for a consensus among genes implicitly requires that at least some individual genes be historically representative" (Olsen & Woese, 1993). Protein-based phylogenies suffer from an insufficient database. A "good" molecule is one that truly reflects the history of its organism and this is determined empirically when a number of proteins mutually confirm the same relationships. These recurring and therefore "true" relationships, and the molecules that predict them, will only be

confidently known when very many molecular sequences have been compared over very many groups of bacteria.

1.2.2 Nucleic Acids

DNA, as the most fundamental of the information-bearing molecules, expresses most completely the genetic and therefore phylogenetic background of the cell. It is, together with its immediate transcripts (RNAs), most independent of environmental influences and changes within it more closely approximate selective neutrality than is possible for its derivative molecules.

1.2.2.1 DNA G+C content

One of the earliest uses of the primary macromolecules in bacterial taxonomy was comparison of the base composition of genomic DNA, expressed as the percentage of guanine and cytosine in the bacterial chromosome. This measure has been shown to maintain a reasonable consistency over generic groups, usually within 10% between species of the same genus (Sueoka, 1961) and less than 5% within species (Mandel, 1969). There are exceptions to these tendencies, for example in the otherwise highly coherent genus *Spirochaeta* (Stackebrandt & Woese, 1981).

Because many unrelated groups of bacteria have a similar G+C base ratio, however, its use as a diagnostic parameter is limited to an exclusivity function: a strain may be eliminated from a certain category on the basis of an incommensurable G+C mol%, but its allocation to a category on the basis of a commensurable one is not definitive. Nevertheless, characterisation by DNA base ratio is a requirement of the minimum list of data for description of new species (International Committee on Systematic Bacteriology. Subcommittee on the Taxonomy of Mollicutes, 1987). When used in conjunction with chemotaxonomic and other molecular characters, DNA base composition is a useful taxonomic marker.

1.2.2.2 DNA-DNA hybridisation

Dissociation of the two strands of a DNA molecule (denaturation) will occur with heating. The strands will reassociate on cooling, re-establishing specific hydrogen-bonds in accord with Watson-Crick base-pairing. Strands will reassociate completely with their fully complementary strands, and less completely with non-homologous strands. Degree of reassociation is reflected in the stability of the double-stranded molecule so formed and can be measured in a number of ways. The technique of DNA-DNA hybridization utilises this principle to measure the degree of nucleotide difference between the chromosomes of two bacteria, expressed as a % similarity or as the difference in melting temperature between the hybrid molecule and the homologous reference molecule (ΔT_m, where T_m is the midpoint of the thermal transition of DNA). It has been estimated that a ΔT_m of 1-2.2°C reflects a sequence dissimilarity of approximately 1% (Britten & Kohne, 1968; Bonner et al., 1973; Ullman & McCarthy, 1973).

DNA-DNA hybridisation has many advantages as a taxonomic tool. Although it is an indirect measure, it is a measure of whole genome difference and as such contains the maximum genetic information available for comparison. "Optimal" experimental conditions, involving hybridisation temperatures 25°C below the melting point of the reference DNA (T_m - 25°C) (Marmur & Doty, 1969; Mandel, 1969; Owen & Pitcher, 1985), ensure that only stable heteroduplices form, and this stability is measured. The elimination of unstable hybrids means that the continuum of characters that has plagued phenotypic definition is avoided: "organisms tend to be closely related or not" (Krieg, 1988). Stable hybrid DNA molecules will form only between complementary strands that have strong sequence similarities, where genomic sequences have no more than 15% mismatch (Johnson, 1991). Therefore, DNA-DNA hybridisation is limited in its taxonomic application to species and genus levels.

As a technique, DNA-DNA hybridisation is relatively easy and cheap to perform (Schleifer & Stackebrandt, 1983; Sneath, 1989; Stackebrandt, 1992). Because it is a binary measure of similarity between two organisms, data are not cumulative and those derived from one comparison cannot be immediately referred to a further comparison.

These limitations notwithstanding, DNA-DNA hybridisation is currently accepted as the most definitive criterion by which bacterial strains are taxonomically recognised. The Ad Hoc Committee on the Reconciliation of Approaches to Bacterial Systematics (Wayne et al., 1987) has defined bacterial species:

"The phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m. Both values must be considered." Although it is not immediately clear from this statement of definition, the term "relatedness" here means the measure of DNA-DNA reassociation and not a measure of genomic sequence homology (Stackebrandt, 1992).

It is less formally accepted that a genus is roughly approximated by a reassociation value of 20% or more under optimal conditions (Johnson, 1984), though this criterion does not always lead to useful classification (Amann et al., 1992). It has been stressed that classification of bacteria into DNA hybridisation groups must be validated by clear phenotypic distinction before nominal definition is made (Wayne et al., 1987). As with other taxonomic data, hybridisation results should be applied to the clarification of hierarchical boundaries with caution and much corroborative evidence.

1.2.2.3 DNA-rRNA hybridisation

A ribosomal RNA cistron is that section of the bacterial chromosome coding tandemly aligned genes for the three different rRNA molecules (16S, 23S and 5S). These genes are separated by non-coding spacer regions (Brosius et al., 1981) which themselves often contain an unrelated gene such as that for a tRNA (Lund et al., 1976). A bacterial genome may have one or several such cistrons (Kohne, 1968; Ryan & Morowitz, 1969). DNA-rRNA hybridisation has been used in phylogenetic analysis on the fundamental premise that rRNA cistrons are more highly conserved than other parts of the genome. The level of relatedness most effectively clarified by this technique is the intergeneric and the supra-generic, making it a very useful complement to DNA-DNA hybridisation which is most effectively applied at the species and intrageneric levels.

Labelled rRNA is hybridized with immobilised single-stranded genomic reference DNA (Schleifer & Stackebrandt, 1983). rRNA-DNA association is based not only on sequence homology as it is for DNA-DNA association, but also on genome size, cistrons per genome and the replicative state of the cell. These factors make analysis on the basis of percentage rRNA-DNA binding an unreliable measure of similarity. Similarity is, therefore, measured as a thermal stability value (T_m) under optimal conditions (Schleifer & Stackebrandt, 1983).

The reduction in sampling error (Sneath, 1988) consequent upon the use of the full 5,000 or so bases of the rRNA cistron (Kohne et al., 1968; Brenner & Falkow, 1971; De Ley et al., 1986) is an advantage of this method. Compared to rRNA cataloguing, it is cheap and cost effective (Kilpper-Bälz, 1991). Results correlate with similarity coefficients above S_{AB} values of 0.45, but data are only approximately comparable (Fox & Stackebrandt, 1987). Phylogenetic reconstructions accord impressively with both complete 5S and 16S sequence analysis at familial and ordinal levels (Sneath, 1988)

1.2.2.4 Nucleic acid sequences

The most straightforward way to classify organisms in accord with the dictum that taxonomy must reflect phylogeny (Wayne et al., 1987) is by comparative molecular sequence analysis. This is because:

* the primary molecules provide much more evolutionary information than their derivative molecules, or their biochemical or morphological effects (Olsen, 1988)

* the sequence itself is extensive and complex. Therefore, evolutionary convergence on any particular configuration of significant length does not occur. Sequence similarity therefore signifies relatedness (Woese et al., 1985b).

* only conserved attributes are useful in establishing genealogical relationships, but when a function is highly conserved, phenotypic variation is minimal. Phylogenetic comparison is only possible in the slow, constrained drift of primary sequence (Olsen & Woese, 1993).

* changes in nucleic acid sequences are largely independent of the selective pressures that act on phenotype. Molecular evolution basically proceeds by selectively neutral random mutations fixed over time, resulting in "clock-like" changes that can convey quantitative measures of phylogenetic relationship (Woese et al., 1985b; Woese 1987a). Extent of divergence can be quantified relatively and branching order of genealogical lineages inferred (Wheelis et al., 1992)

* common ancestry of compared characters is the critical basis for phylogeny. Simple, unambiguous characters allow objective recognition of genuine homology (Gutell et al., 1985; Olsen, 1987).

* comparative data is cumulative and can be handled mathematically across large data sets.

1.2.2.4.1 16S rRNA - the "properly constrained molecule"

All molecular sequences are not equally valuable in determining phylogenetic relationships. Molecules must have certain attributes before comparison of their sequences are phylogenetically meaningful. Fox et al. (1980) in their classic paper referred to ribosomal RNA as "the properly constrained molecule": the molecule which, because of the many and various restrictions upon its evolution, most accurately reflects the phylogenetic history of an organism. The features of the 16S rRNA molecule that so befit it for its phylogenetic role are:

1. *universality*

The translation mechanism to which the ribosome is central was established early in evolution (Fox et al., 1980). It is consequently universal and its components are therefore comparable across all organisms, circumscribing a phylogeny that encompasses the three primary kingdoms (Woese & Fox, 1977).

2. *functional constraints*

The premise that all ribosomal RNA is functionally equivalent, while not provable (Woese et al., 1985c), is a reasonable assumption. Primary sequences are largely identical (Woese et al., 1975), secondary structures are equivalent (Gutell et al., 1985; Neefs et al., 1990) and various constituents of the ribosome (including rRNA) can be exchanged between organisms to form functional heterologous ribosomes (Nomura et al., 1968). Structural conservation reflects conservation of the complex and precise function of the translation apparatus.

3. *selectively neutral evolution*

A second reasonable assumption, though also not provable (Woese et al., 1985c), is that sequence changes in rRNA are selectively neutral, the result of random mutations fixed in a population by genetic drift. Selected, non-isochronic changes are rare (Woese 1985b). Because no post-transcriptional modifications are made to ribosomal transcripts, rRNA sequence exactly reflects primary DNA sequence. It is effectively a primary semantide and as such is maximally independent of organismal phenotype and therefore selection pressure (Woese et al., 1985a). rRNA analysis also avoids the complication of codon degeneracy that depreciates information content, obscures homology and impedes analysis in protein-coding genes.

4. *constraints on horizontal genetic transfer*

A third necessary and reasonable assumption (Woese, 1992) is that there is no lateral transfer between organisms of genes coding the ribosomal transcripts, and that these molecules truly represent the history of their organisms. Because the ribosome is a large molecular complex intrinsic to cell function, lateral transfer is less likely than for more peripheral cell functions (Woese, 1992). The phylogenetic relationships derived from 16S rRNA sequences have been confirmed by independent analysis of other gene products (Olsen & Woese, 1993).

5. *size*

Molecular size is an inevitable, though not sole, determinant of information content (Olsen & Woese, 1993). Larger molecules usually provide more precise and reliable phylogenetic information and serve as less erratic chronometers than smaller molecules. 16S rRNA is large enough to give statistically meaningful analyses. It is approximately 13 times the size of 5S rRNA, and half the size of 23S rRNA. The 5S molecule has been shown to be too short for resolution of ancient genealogical relationships and too conserved for resolution of recently evolved ones. Phylogenetic trees derived from 5S and 16S rRNA molecules differ significantly at all taxonomic levels (Stackebrandt, 1992). The average rate of sequence change in the 23S rRNA gene is twice that for the 16S rRNA gene; consequently any information advantage due to its larger size is negated. Its usefulness is not proportional to its length. Phylogenies derived from 16S and 23S rRNA sequences are very similar (Olsen & Woese, 1993).

The conformation of the 16S rRNA molecule describes about 50 domains: helices and loops that maintain the functional integrity of the molecule. The effects of any (albeit rare) non-chronometric alteration in sequence will be localised within one of these domains, restricting the impact of such a change on the molecule as a whole (Woese et al., 1985b). The 5S rRNA molecule, on the other hand, has only five domains and such selective effects will be proportionately more pronounced (Woese et al., 1976; Weisburg et al., 1989)

6. *differential constraints*

Over the length of the 16S rRNA molecule regions of sequence are conserved to different degrees. Some sequences are invariant in all molecules; some are variable even over short genetic distances (Woese 1987). Nucleotide replacement frequencies at various positions in the 16S rRNA sequence differ by more than 100-fold (Gupta et al., 1983). Highly conserved regions correspond to functional centres where secondary structure must be maintained or interactions with other translational components occur (Nomura et al., 1969). In phylogenetic analysis, these differences can be exploited to describe relationships at different

taxonomic levels and correspond, in effect, to molecules with differing rates of evolution.

Regions of invariant sequence and higher order structural similarity across taxa (Woese et al., 1983) allow sequence homologies to be established with confidence and extended to variable regions during alignment (Gutell et al., 1985; Olsen et al., 1986; Olsen, 1987).

7. *isochronic evolution*

With a few notable exceptions, rRNA has apparently evolved at the same rate in all bacterial lineages (Ochram & Wilson, 1987), consistent with the chronometric behaviour of neutral mutations (Kimura, 1983). This allows the estimation of the phylogenetic depth, or age, of a particular line of descent and definition of the order in which lineages diverged (Olsen, 1987). Bacterial groups, such as the mycoplasmas, that depart from this isochronicity are readily identified, not only by exaggerated sequence divergence in 16S rRNA genes but by the abnormal distribution of these differences throughout normally well-conserved regions (Woese et al., 1985c). Such "tachytelic" evolution appears to result from an elevated mutation rate (Ghosh et al., 1977), and is consistent with the theoretical basis of chronometric evolution.

8. *accessibility*

The 16S rRNA molecule occurs in about 10^4 copies per logarithmic phase cell. It is accessible, therefore, for reverse transcription and subsequent sequencing. The regions of sequence invariance within the gene make it amenable to selective amplification by polymerase chain reaction using primers to terminal conserved regions with subsequent direct or clone-based sequencing.

1.2.2.4.2 rRNA catalogues

Phylogenetic analysis of partial rRNA sequences by the method of "cataloguing" has been applied to each of the bacterial rRNA molecules (Sogin et al., 1972; Fox & Stackebrandt, 1987), but most extensively and successfully to the 16S rRNA component of the small ribosomal subunit.

Although with technological advances cataloguing has been superseded by full sequence comparisons, bacterial phylogeny was founded on partial sequence data (Fox et al., 1980; Woese, 1987) and an extensive and valuable literary corpus exists based upon 16S rRNA catalogues.

Purified 16S rRNA is digested with the guanosine-specific nuclease T_1 and fragments are separated by thin layer chromatography (Stackebrandt et al., 1981). Each oligonucleotide is then sequenced. This produces 70-80 oligonucleotide sequences (Martens et al., 1987) of 6-20 nucleotides on average, collectively referred to as a catalogue (Fox & Stackebrandt, 1987). Comparisons, therefore, involve approximately 35-40% of the total sequence of the molecule (Martens et al., 1987). This is effectively representative insofar as fragments are randomly generated from the molecule's entire length. Because guanosine is the nuclease substrate, regions of high G content may be lost, and bias may be introduced in analysis of strains with an overall high G+C genome content. Such potential distortions, however, have not had great impact on the results.

Binary comparisons are made of catalogues to establish the "similarity coefficient" S_{AB}, using the equation (Fox et al., 1977):

$$S_{AB} = 2N_{AB} / N_A + N_B$$

where N_A is total residues in oligonucleotides of at least minimum length in catalogue A, N_B is the comparable measure for catalogue B, and N_{AB} is total residues contained within all coincident oligonucleotides of minimum length between the two catalogues. Fragments of less than 6 bases are ignored in computations for statistical reasons except for very closely related organisms (Fox et al., 1977). From a matrix of S_{AB} values a dendrogram is derived by average linkage clustering (Anderberg, 1973).

Phylogenies and classifications established by 16S rRNA catalogue analysis have needed very little revision with subsequent access to full 16S rRNA sequences. However, 16S rRNA sequence similarity values and binary similarity coefficients correlate in a non-linear way that cannot be derived theoretically. Nor can either parameter be predicted from the other (Stackebrandt, 1988). S_{AB} values underestimate relatedness over the whole range of values but especially in ancient lineages. Correlation between binary similarity coefficients and DNA-DNA hybridisation data are also non-linear. S_{AB} values for two organisms must be at least 0.75 to expect DNA homologies of more than 30% (Stackebrandt, 1988).

Mathematical correlations have been attempted over a subset of $S_{AB}>0.3$, which excludes distantly related organisms (Devereux et al., 1990). Nonconformities arise because, while roughly representative of the whole gene, catalogue data inevitably contains less information and will underestimate (or fail to pick up at all) small differences between closely related groups and also underestimate the larger differences between distantly related groups in proportion to the degree of that distance. Phylogenetic resolution within ancient genealogical lineages is low. Consequently, strict application of S_{AB} values to precisely define taxa often transgresses the hierarchical clustering of groups currently recognised on many different criteria (Fox & Stackebrandt, 1987).

1.2.2.4.3 signatures

With compilation of catalogues representing very many bacterial strains, recurrent sequence motifs and structures have been recognised that are unique (or effectively so) to a particular taxon (Woese et al., 1985b; Woese, 1987). These are oligonucleotide "signatures", characters within the sequence of the 16S rRNA gene that can be used to define a bacterial group. There is no necessary recourse to binary analysis. Conventional sequence analyses address each sequence position as of equal phylogenetic importance; signature analysis exploits the frequency and pattern of sequence changes at certain specific positions in the 16S rRNA molecule to define phylogenetic taxa (Woese et al., 1985a). Therefore, more historical information can be accessed, making signatures the most useful aspect of partial rRNA sequence data (Woese et al., 1985b)

1.2.2.4.4 full 16S rRNA sequences

Technological advances now facilitate easy access to full 16S rDNA sequences. The advantages entailed include:

* replacement of binary analyses with matrix algorithms
* sequence comparisons can be accumulated and new sequence added into an existing comparison
* maximum phylogenetic information can be retrieved from the molecule

* analysis of full sequence data increases the statistical validity of computations and makes the molecule a more reliable chronometer (Woese, 1987b)
* phylogenetic relatedness at all taxonomic levels can be established by comparing regions of different variability (Witt & Stackebrandt, 1990)
* knowledge of the primary and secondary structures of the rRNA molecule gives insights into its evolution that are invaluable in taxonomic assessment (Woese et al., 1983).

Some authors have suggested that 16S rRNA sequence comparison measures can be correlated with measures of genomic similarity in a numerically meaningful way. Chromosomal reassociation values of 50% have been given as corresponding to 99% 16S rDNA sequence similarity for *Desulfovibrio* and other published sequences (Devereux et al., 1990) or to 98% 16S rDNA sequence similarity for *Fibrobacter* strains (Amann et al., 1992). However, the level of 16S rRNA sequence similarity among members of genera can itself vary widely. Species of the genus *Streptomyces* have a similarity level of >95% in their 16S rRNA sequences; species of spirochaetes 78% (Stackebrandt, 1988). Others authors suggest that the relationship itself is not predictable within all taxonomic groups (Fox et al., 1992; Martinez-Murcia et al., 1992a).

1.2.2.4.5 inferring phylogenetic trees

There are two basic methods for inferring phylogenetic trees from sequence data: evolutionary distance analysis and maximum parsimony analysis (Woese, 1992).

Evolutionary distance - is a pairwise comparison of the number of differences in two sequences expressed as the "distance" between the sequences (Fitch & Margoliash, 1967). The actual number of differences is inevitably underestimated because multiple changes at a position go unrecognised. This underestimation increases over greater actual genetic distance but statistical corrections can be made (Jukes & Cantor, 1969). A least-squares analysis accommodates the disagreement between distance estimates and any given additive tree to define branching order and branch lengths most consistent with the data (Olsen, 1988).

Maximum parsimony - examines sequence differences individually and postulates the minimum number of sequence changes necessary to derive one sequence from another (Fitch 1971). This method also overlooks superimposed substitutions, with consequent overestimation of relationship. While there is no explicit correction for multiple substitutions in maximum parsimony analysis (Felsenstein, 1982), systematic error arising in this way can be minimised by comparing sequence sites with the lowest rates of nucleotide substitution (Felsenstein, 1978).

There are many variations and modifications to these algorithms. Each is subject to systematic errors as well as the random errors intrinsic to all phylogenetic trees (Olsen & Woese, 1993). Different trees can be constructed by applying different algorithms to the same data set (Saitou & Nei, 1987). Attention to the assumptions underlying each method enhances its usefulness (Olsen, 1988). However, it is suggested that the relative merits of different mathematical approaches for inferring trees are of less importance than the validity for phylogenetic inference of the sequences to which they are applied (Olsen & Woese, 1993).

1.3. Summary

The hierarchical ordering of bacteria on the basis of natural evolutionary relationships has been made possible with the use of comparative molecular data. Analysis of molecules has provided the framework for this hierarchy and insight into the two distinctive characters of the evolutionary process: genealogy (branching order) can be inferred quite precisely; and the extent of divergence between groups can be quantified at least in relative terms (Wheelis et al., 1992).

Taxa at each level of the hierarchy describe monophyletic groupings and the degree of relationship between these groups basically indicates taxonomic rank. "Species", alone, has been formally defined in terms of molecular comparisons. Taxonomic ranks above and including "genus" are less amenable to inflexible definition by molecular parameters. There are currently no phylogenetic definitions applied to them and the scope of any such definition may differ between taxa of the same rank (Wayne et

al., 1987). Higher taxonomic ranks must be defined by a broad range of 18phylogenetic parameters (Fox & Stackebrandt, 1987). Even the definition of species in terms of DNA-DNA reassociation measure is the basis of a polyphasic taxonomy. It is fundamental that genetic distinction of species be corroborated by phenotypic distinction (Wayne et al., 1987).

2. BACTERIAL STRAINS

2.1. *Paracoccus halodenitrificans*

2.1.1 Genus *Paracoccus*

The genus *Paracoccus* was described initially to accommodate the two species *P. denitrificans* (Beijerinck 1910) Davis 1969 ATCC 17741^T and *P. halodenitrificans* (Robinson & Gibbons 1952) Davis 1969 (Kocur, 1984). Both species are non-motile cocci or rods, oxidase- and catalase-positive, with optimal temperatures for growth of 25-30°C, which accumulate intracellular granules of poly-β-hydroxybutyrate. Both are facultatively anaerobic if nitrate, nitrite or nitrous oxide are present as terminal electron acceptors (Kluyver, 1956). The ultrastructures of the two species are similar (Kocur et al., 1968). The species differ insofar as *P. denitrificans* is a non-halophilic autotroph in the presence molecular hydrogen, while *P. halodenitrificans* is a non-autotrophic halophile (van Verseveld & Stouthamer, 1991).

2.1.2 *Paracoccus halodenitrificans*

P. halodenitrificans is a gram-negative coccus or rod (0.5μm in diameter or 1.2μm long), which occurs singly or in pairs. It has a specific NaCl requirement, with growth over the range 2.2 - 20% (optimally 4.4 - 8.8%), and is facultatively anaerobic. The species was first described as *Micrococcus halodenitrificans* in 1952 (Robinson & Gibbons) after isolation from meat curing brine. Only one strain has been recorded (DSM 735^T = CCM 286^T = NCMB 700^T = ATCC 25843^T = ATCC 13511 [Urakami et al., 1990]).

2.1.3 *Paracoccus* taxonomy

The type species *P. denitrificans* has been taxonomically aligned by several criteria with the purple photosynthetic bacteria (Woese et al., 1984), now referred to as the class Proteobacteria (Stackebrandt et al., 1988). 16S ribosomal RNA sequence catalogue analysis places *P. denitrificans* close to *Rhodopseudomonas sphaeroides* in the α-subclass of the Proteobacteria (Woese et al., 1984) which constitutes the family

Rhodospirillaceae (Pfennig & Trüper, 1971; Imhoff & Trüper, 1991), formerly the purple, non-sulphur bacteria. (Only one genus of the purple non-sulphur bacteria, *Rhodocyclus*, falls outside this classification and into the β-subclass of the Proteobacteria [Imhoff & Trüper, 1991]).

Both *P. denitrificans* and *Rsp. sphaeroides* have been shown to undergo an unusual and specific cleavage of the 23S rRNA transcript, giving rise to a "14S" and a "16S" fragment which are then both incorporated into the large 50S subunit of the functional ribosome (MacKay et al., 1979). T1 oligonucleotide catalogue analysis of the 14S fragment shows it to correspond to the 5'-terminal section of the 23S rRNA precursor and identifies a high level of sequence homology between the two species (S_{AB} = 0.47). This data is supported by similar findings using 16S rRNA catalogue data (Fox et al, 1980) which relates the two type species at an S_{AB} of 0.66 (consistent with the observation that sequence conservation in 16S rRNA catalogues is higher than in 23S rRNA catalogues [Woese et al., 1976]). The unusual cleavage of the 23S rRNA transcript giving rise to fragments of these particular sizes has only been described for two other species, *Agrobacterium tumefaciens* (Grienenberger & Simon, 1975; Schuch & Loening, 1975) and *Rhodopseudomonas capsulata* (Lessie, 1965), also members of the α-subclass of the Proteobacteria. Sequence comparisons the 5S rRNA molecule of *P. denitrificans* and that of *Rhodospirillum rubrum* (MacKay et al., 1982) indicate a strong relatedness, with only 22% nucleotide differences, strengthening the assignment of *P. denitrificans* and the genus *Paracoccus* within the Rhodospirillaceae.

Structural similarities and comparable amino acid sequences of the c-type cytochromes of *P. denitrificans* and other members of the Rhodospirillaceae (John & Whatley, 1975; Timkovich & Dickerson, 1976) support the nucleic acid evidence of their phylogenetic proximity. (*Rhodopseudomonas sphaeroides* was reclassified as *Rhodobacter sphaeroides* during the course of the references cited herein, on the basis of 16S rRNA catalogue analysis [Imhoff et al., 1984]). More recent comparative analysis of complete 16S rRNA sequences (Ludwig et. al., 1993) places *P. denitrificans* in the α-subclass, most closely related to *Thiobacillus versutus* and members of the genus *Rhodobacter,* including *Rh. sphaeroides.*

The phylogenetic relationship between *P. denitrificans* and *P. halodenitrificans* has not been established by molecular criteria, and is based solely on phenotypic similarities and comparable G+C content of their DNA (van Verseveld & Stouthamer, 1991). *P. halodenitrificans* is placed in the α-subclass of the Proteobacteria by generic association.

2.1.4 Classification of *P. halodenitrificans*

Since its establishment, several new species have been added to the genus *Paracoccus*: *P. alcaliphilus, P. aminophilus, P. aminovorans* and *P. kocurri* (Urakami et al., 1989; Urakami et al., 1990; Ohara et al., 1990). Examination of the traits common to this larger group has suggested strongly that *P. halodenitrificans* is misplaced in this genus.

Cellular fatty acid profiles of the group show a preponderance of $C_{18:1}$, except for *P. halodenitrificans* which contains large amounts of $C_{16:0}$, $C_{18:1}$ and, uniquely for the genus, $C_{16:1}$. The major hydroxy fatty acids common to the genus are 3-OH $C_{10:0}$ and 3-OH $C_{14:0}$; *P. halodenitrificans* contains only 3-OH $C_{12:0}$. The major respiratory quinone utilised by members of the genus *Paracoccus* is ubiquinone 10, with ubiquinones 8, 9 or 11 occurring as very minor components. *P. halodenitrificans*, in contrast, uses ubiquinone 9, with very low levels of both ubiquinones 8 and 10. (Urakami et al. 1985, 1989, 1990; Urakami & Komagata, 1986, 1987).

When genomic DNA of *P. halodenitrificans* was hybridised with that from strains since described as *P. aminovorans* and *P. aminophilus* (Urakami et al., 1990), reassociation data indicated that congeneric classification was dubious. Both new strains were significantly more closely related to the type species *P. denitrificans* (52% and 30% DNA-DNA reassociation, respectively) than to *P. halodenitrificans* (10% and 11% DNA-DNA reassociation, respectively).

The synthesis of polyamines by the six recognised species of *Paracoccus* was scrutinised (Hamana & Matsuzaki, 1992). Five species contain spermidine and putrescine as major polyamines, and ubiquitously produce norspermidine from supplemented diaminopropane. Some species also produce two aminopropyl derivatives of cadaverine, aminopropylcadaverine and aminopentylnorspermidine. The biosynthesis of these unusual polyamines was proposed as a chemotaxonomic marker

for the genus *Paracoccus*. Conspicuously, *P. halodenitrificans* contains only spermidine as its major polyamine with no putrescine present, and does not synthesise norspermidine nor any cadaverine derivatives. The authors have suggested that *P. halodenitrificans* is not a valid member of the genus *Paracoccus*.

Regarding the generic misplacement of *P. halodenitrificans*, these various chemotaxonomic data are independently convincing and collectively near-conclusive. However, direct phylogenetic evidence based on comparative 16S rRNA sequence analysis is warranted to test the hypothesis of misclassification and establish the appropriate taxonomic position of *P. halodenitrificans*.

2.1.5 Family Halomonadaceae

There are phenotypic, chemotaxonomic and molecular characteristics shared by members of the family Halomonadaceae and *P. halodenitrificans*.

The two species *Halomonas* and *Deleya* constitute the family Halomonadaceae, which belongs in the γ-subclass of the Proteobacteria (Franzmann et al., 1988). All members are Gram-negative rods, with a requirement for NaCl and a wide range of halotolerance. G+C DNA content ranges between 52-68 mol%. An ability to accumulate poly-β-hydroxybutyrate granules typifies species of the genus *Deleya* (Kersters, 1991). These characteristics are also typical of *P. halodenitrificans*. In the description of a new species of *Halomonas*, *H. subglaciescola* (Franzmann et al, 1987), numerical analysis of 92 phenotypic traits placed the new species equidistant between *H. elongata* and *P. halodenitrificans*. 16S catalogue analysis placed *H. subglaciescola* in the family Halomonadaceae (Franzmann et al. 1988). These data together imply that the species *P. halodenitrificans* is better accommodated within the Halomonadaceae.

Chemotaxonomic comparisons further support the re-classification of *P. halodenitrificans*. Members of the Halomonadaceae use ubiquinone 9 as the major respiratory quinone, with traces of ubiquinone 8 (Franzmann & Tindall, 1990). Major fatty acids are $C_{18:1}$, $C_{16:0}$ and $C_{16:1}$ (Franzmann & Tindall, 1990; Skerratt et al., 1991), and $C_{cy19:0}$ and $C_{cy17:0}$ under certain conditions (Franzmann & Tindall, 1990). Apart from the presence of

cyclopropane fatty acids, which has not been reported for *P. halodenitrificans*, the chemotaxonomic profiles of the Halomonadaceae are consistent with that of *P. halodenitrificans*.

The data described above indicate that *P. halodenitrificans* does not belong in the genus *Paracoccus*, but could well be comfortably accommodated in the family Halomonadaceae. A comparison of 16S rRNA sequences is made which includes that of *P. denitrificans* (Ludwig et al., 1993) and those of several species from the genera *Deleya* and *Halomonas*.

2.2 *Aeromonas salmonicida*

2.2.1 Genus *Aeromonas*

The genus *Aeromonas* Kluyver and Niel (1936) circumscribes a number of oxidase- and catalase-positive, facultatively anaerobic gram-negative rods. It has classically been divided into the motile mesophilic aeromonads and the psychrophilic non-motile aeromonads. The latter are contained within the single species *A. salmonicida*. Much data exists suggesting that the motile aeromonads constitute a diverse group of species, while *A. salmonicida* is a unit with high genetic integrity (MacInnes et al, 1979; Belland & Trust, 1988; Hennigan et al., 1989; Martinetti Lucchini & Altwegg, 1992; Martinez-Murcia et al., 1992a). DNA relatedness studies indicate that the genus can be divided into 13 hybridisation groups (Popoff et al., 1981; Kuipjer et al, 1989; Hickman-Brenner et al., 1987 & 1988; Schubert, 1990), currently recognised as the species *A. hydrophila, A. salmonicida, A. caviae, A. sobria, A. media, A. eucrenophila, A. schubertii, A.veronii, A. jandaei, A. trota, A. enteropelogenes* and *A. ichthiosmia* (Martinez-Murcia et al., 1992a), and *A. allosaccharophila* (Martinez-Murcia et al., 1992b). Members of the genus are ubiquitously distributed, with many significant pathogens occurring among them.

The genus *Aeromonas* was originally included in the eubacterial family Vibrionaceae. However, convincing molecular data including 5S and 16S rRNA gene sequences and DNA-DNA hybridisation studies have identified the need to establish a family Aeromonadaceae to accommodate the genus (Colwell et al., 1986).

2.2.2 *Aeromonas salmonicida*

Species description

Aeromonas salmonicida is a Gram negative rod, non-motile and fermentative, which produces a soluble brown pigment and grows optimally between 10-15°C, but not at 37°C (Farmer et al., 1991). It has been recognised since its isolation in 1894 as the aetiological agent of the disease furunculosis in salmonid fish (Emmerich & Weibel, 1894), and as such is one of the earliest described fish pathogens (Egidius, 1984). *A. salmonicida* appears to have derived originally from Europe and has since spread globally (Austin & Austin, 1987). It was first described in Australia in 1980 in association with ulcerative disease in goldfish (Trust et al., 1980).

Easy identification of *A. salmonicida* has been hampered by lack of a definitive selective medium, its slow growth characteristics and poor plating efficiency in mixed culture which confer upon it a tendency to be out-competed by other species (Gustafson et al., 1992). These difficulties in culturing the pathogen may have contributed to its (perhaps gratuitous) reputation as an obligate parasite (Hiney et al., 1992) and have hindered study of its ecology and epidemiology.

Pathogenicity

The disease furunculosis is responsible for severe economic losses in the farmed fish industry world-wide. The disease itself occurs as a highly communicable septicaemia (Farmer et al., 1991) with three principal manifestations. Sub-acute furunculosis, sometimes referred to as chronic furunculosis, produces boil-like furuncles on the muscle tissue. It occurs most frequently in older fish and is not usually fatal. Acute furunculosis has few symptoms but a rapid and extensive mortality. The acute form of the disease is most common, and primarily affects growing fish and young adults with consequent pronounced effects on the fishing industry. Peracute furunculosis affects fingerlings, with mild symptoms and high mortalities, resulting in serious industrial losses (Davis, 1946).

With infection the pathogen is found in the blood, throughout the tissues, within the lesions of the chronically-infected fish, in the waters of the

fishes' environment and the sediments below these (O'Brien et al., 1994). Although the extent to which the bacterium can survive outside the host is still unclear, and data on free-living bacteria have been conflicting (Allen-Austin et al., 1984; Rose et al., 1990a; Rose et al., 1990b; Morgan et al., 1993), some viability does seem to be maintained for a significant time in the environment. Outbreaks of furunculosis have been shown to correlate with elevation of water temperatures (Egidius, 1984).

Furunculosis has not been reported in Australia (Humphrey et al., 1987). However, an endemic atypical strain of *A. salmonicida*, associated with goldfish ulcer disease, has been shown experimentally to cause acute septicaemia in Atlantic salmon (Carson & Handlinger, 1988).

Typical and atypical *A. salmonicida*

The host range of the bacterium was initially considered to be restricted to salmonids: Atlantic salmon *Salmo salar* (Paterson et al., 1980a), brown trout *Salmo trutta*, brook trout *Salvelinus fontinalis*, coho (silver) salmon *Oncorhynchus kisutch*, chinook salmon *On. tshawytscha*, rainbow trout *On. mykiss*, sakuramasu *On. masou*, pink salmon *On. gorbuscha*, both with and without associated pathology (Austin & Austin, 1987 and refs. therein). Atlantic salmon are more sensitive to the pathogen than are rainbow trout which are more likely to become asymptomatic carriers (Fish, 1937; Carson, 1990).

With time and ongoing research *A. salmonicida* has been isolated from several different genera of farmed and wild fish including goldfish *Carassius auratus* (Mawdesley-Thomas, 1969; Elliott & Schotts, 1980; Trust et al., 1980), bream *Abramis brama*, roach *Rutilis rutilis*, tench *Tinca tinca*, dace *Leuciscus leuciscus* (Carson, 1990), turbot *Psetta maxima* and wrasse *Ctenolabrus rupestris* (Carson, 1990) and sablefish *Anoplopoma fimbria* (Evelyn, 1971). The disease manifested in these instances is slightly different from classical furunculosis, most commonly appearing as chronic ulcerative infections. The bacteria involved also differ biochemically in several ways from *A. salmonicida* as described, though insufficiently for them to be designated separate species. These differences have evoked a semantic distinction: strains giving rise to non-salmonid ulcerative diseases have been called "atypical" *A. salmonicida* (McCarthy, 1980) in acknowledgement of the variations in host, in disease

symptoms, in culture requirements and biochemical variability, usually including absence of pigment (Austin & Austin, 1987). Subsequently, however, atypical strains have been shown to also infect salmonid host species (Evelyn, 1971; Paterson et al., 1980a).

Early descriptions of *A. salmonicida* noted the brown water-soluble pigment produced by the bacterium on media containing tyrosine or phenylalanine (Griffin et al. 1953; O'Leary et al. 1956). Although pigment production is designated a distinguishing characteristic of the species (Popoff 1984), not all strains of the bacterium produce pigment (Duff & Stewart, 1933; Evelyn, 1971) and even pigment-producing strains vary in the amount of pigment produced and the temporal predictability of its production (Horne, 1928; Mackie & Menzies, 1938). This is particularly true of strains isolated from non-salmonid hosts ("atypical" strains), though not exclusively so (McCarthy, 1980, for "atypical" pigment-producing strains; Duff & Stewart, 1933; Evelyn, 1971, for "typical" non-pigment producing strains).

A. salmonicida displays a dimorphism in colony type. "Rough" colonies have been shown by electron microscopy to differ from "smooth" colonies in having an additional surface layer, the A-layer, external to the normal outer membrane of gram negative bacteria. (There also exists a state recognised as intermediate between these two called the G-phase [Duff, 1937]) This layer has been implicated in virulence (Udey & Fryer, 1978) and in the strong auto-agglutination of bacteria with this colony type thought to correlate with an ability to adhere to fish tissue (Saikai 1986). In at least some instances, the A-layer can be inhibited by growth on lithium (Duff 1937, Hamilton et al., 1981) or growth at high temperatures (Ishiguro et al., 1981). Recent observations of virulent, auto-agglutinating strains with no obvious A-layer and avirulent strains that possess an A-layer have compromised this correlation (Johnson et al. 1985; Ward et al. 1985).

Intra-species Taxonomy

Semantic distinction of the two groups of *A. salmonicida* was followed by a formal classificatory separation based on numerical taxonomy, wherein non-pigmented atypical forms were assigned to the subspecies *A. salmonicida* subsp. *achromogenes*, NCMB 1110^T (Smith, 1963) or *A.*

salmonicida subsp. *masoucida*, NCMB 2020^T (= ATCC27013^T) (Kimura, 1969a), and the typical strains to the type strain *A. salmonicida* subsp. *salmonicida*, NCMB 1102^T (Schubert, 1974). Much debate has since ensued over the fine distinctions among the atypical *A. salmonicida* strains, principally centred on the distinction between those that infect salmonids and those that infect non-salmonids. However, the overall homogeneity of the species, including typical and atypical strains, has been demonstrated by DNA-DNA hybridisation data (MacInnes et al., 1979). These data suggest a closeness of genetic relatedness that would even preclude any division at subspecies level. The authors suggest that phenotypic variation within the species is attributable to mutant forms of the type strain.

Subsequently, DNA-DNA reassociation analysis (Belland & Trust, 1988) using the same S1 nuclease/TCA technique and G+C mol% data as MacInnes et al. (1979) was applied to closer scrutiny of the genetic structure of the species *A. salmonicida*. A definite genetic distance was discernible between typical strains and atypical strains derived from non-salmonid sources. Typical strains cluster with <1% sequence divergence. Atypical strains are genetically distinct from the typical cluster but do not form an homogeneous grouping among themselves. *A. salmonicida* subspp. *achromogenes* and *masoucida* cluster together and more closely to *A. hydrophila* and the motile aeromonads than to other *A. salmonicida* strains, as was suggested previously (Paterson et al., 1980b). The remaining atypical non-salmonid strains form a group equidistant between the typical and *masoucida* groups, and internal genetic distinctions seem to correspond to the fish genus from which the isolate was taken (either carp or goldfish). Reconsideration of the erstwhile appellation "subsp. *nova* " (McCarthy & Roberts, 1980) to describe this group was recommended, but isolates that seem to correspond, at least in part, have subsequently been incorporated into subspecies *smithia* (Austin et al., 1989). *A. salmonicida* subsp. *smithia*, CCM4103^T, has superseded subspecies "*nova*", (an heterogeneous assembly of strains never formally recognised), subsuming some of the atypical strains from non-salmonid sources.

Much serological data (reviewed in Austin & Austin, 1987), though not always completely consistent, still corroborate the tight integrity of the species with little distinction between the typical and atypical strains. Bacteriophage typing (Paterson et al., 1980b; Popoff, 1984) supports the

extremely coherent clustering of all strains within the species, detecting no differences in sensitivity between chromogenic and achromogenic strains, nor between aggregating and non-aggregating strains. *A. salmonicida* phages are exclusively species specific (with the proviso that the species *Haemophilus piscium* be re-classified as an atypical, achromogenic strain of *A. salmonicida*).

2.2.3 *Aeromonas salmonicida* in Australia

The diseases carp erythrodermatitis (CE) and goldfish ulcerative disease (GUD) are both characterised by chronic cutanaceous ulcerative lesions (Fijan, 1972; Elliott & Schotts, 1980). Both have been attributed to infection by *A. salmonicida*, though the biovars implicated bear significant phenotypic differences from recognised atypical subspecies *masoucida* and *achromogenes* (Popoff, 1984; Skerman et al., 1980). These strains are phenotypically characteristic of the subspecies *smithia*.

Both diseases are well documented globally. As early as 1969, furunculosis or dermal ulceration was reported in goldfish and other non-salmonid fish in Australia (Mawdesley-Thomas, 1969). The aetiological agent was presumed to be *A. hydrophila*, as *A. salmonicida* was then considered to be host specific. These misconceptions were reinforced by the actual presence of *A. hydrophila* within the lesions of infected fish and by the notorious slow growth habit of *A. salmonicida* that has often led to its oversight. Substantive diagnosis was made in 1977 (Trust et al., 1980). Since that time, outbreaks of GUD have been attributed to atypical *A. salmonicida* in five Australian states (Humphrey & Ashburner, 1993) and it is now considered endemic to south eastern Australia (Carson & Handlinger, 1988), excluding Tasmania (Humphrey & Ashburner, 1993; Carson, 1994). All biochemical, phenotypic and protein comparisons (Whittington et al., 1987) suggest that the strain was introduced to Australia with ornamental goldfish from Japan.

Experimental challenge with the endemic Australian GUD-inducing strains against Atlantic salmon (*Salmo salar* L.) gave rise to typically acute septicaemia, with severe mortality (LD_{50} of three cfu in parenterally challenged fish), indicating that Atlantic salmon is a highly susceptible host to this biovar (Carson & Handlinger, 1988; Whittington & Cullis, 1988).

Atypical *A. salmonicida* derived from CE infected cyprinids was found to invoke a less virulent response in rainbow trout (Obradovic, 1983).

2.2.4 *A. salmonicida* strain 93/1061

The first isolation in Australia of an atypical *A. salmonicida* from a greenback flounder (*Rhombosolea tapirina*) occurred in March, 1993 in a population of farmed fish in northern Tasmania. Pathogenesis involved slowly developing skin lesions. Gram negative coccobacilli were subsequently isolated from kidney and skin, and from other internal organs as infection progressed (J. Carson, pers. comm.).

An atypical strain of *A. salmonicida* has been isolated from flounder (*Platichthys flesus* L.) in the Baltic Sea and Norway (Wiklund & Bylund, 1991; Wiklund et al., 1994), which is cytochrome-oxidase negative. This trait, together with other biochemical anomalies, exclude the strain from currently nominated subspecies. Atypical biovars of *A. salmonicida* have been isolated frequently from species of flatfish in Europe (Wiklund & Bylund, 1991; Carson, 1994).

The Tasmanian bacterium was identified initially as an atypical *A. salmonicida* on the basis of general phenotype. It was further distinguished by restriction fragment length polymorphisms (Whittington et al., 1995) from atypical Australian isolates of *A. salmonicida* taken from pathologically infected goldfish (*Carassius auratus*) and silver perch (*Bidyanus bidyanus*). RFLPs of goldfish and perch isolates showed only minor differences; flounder isolates were clearly distinguishable from both.

There appears to be sufficient data to suggest that strain 93/1061 is a new subspecies of *A. salmonicida* naturally endemic to Tasmanian coastal waters. The taxonomic position of this strain is investigated here using comparative 16S rRNA sequences.

2.3 *Enterococcus seriolicida*

2.3.1 *Enterococcus seriolicida*

Species description

Enterococcus seriolicida was first described in 1991 (Kusuda et al.) after its identification in Japan as the aetiological agent of streptococcicosis in yellowtail (*Seriola quinqueradiata*) and eel (*Anguilla japonica*). Various strains isolated from kidney, liver and/or heart of both fish species all showed the same characteristics by which the species is described (Kusuda et al., 1991). The type strain of the species, YT-3 (= ATCC 49156^T), was isolated from kidney tissue of a Japanese yellowtail in 1974.

Ent. seriolicida is a Gram-positive, catalase-negative bacterium occurring as short chains of ovoid cells elongated with the axis of the chain (1.4 x 0.7μm). It is a non-motile, facultative anaerobe. Morphological and biochemical traits prompted the authors to assign the bacterium to the genus *Enterococcus* (Kusuda et al., 1991). The DNA of the type strain has a G+C content of 44 mol%; the enterococci 37-45 mol%. Several characteristics of *Ent. seriolicida* as originally defined are noteworthy in light of the contention that has subsequently ensued.

1. Unlike other members of the genus *Enterococcus*, *Ent. seriolicida* does not react positively for Lancefield group D antigen. Reaction with group D antisera characterises the revised genus (Schleifer & Kilpper-Bälz, 1984). However, it is now recognised that most but not all enterococci are positive for this antigen (Devriese et al., 1993). *Ent. seriolicida* is also negative for Lancefield group N antigen (and A, B, C, E, F, G, H, K, L, M and O).

2. Like the other enterococci, the species description of *Ent. seriolicida* stipulates growth at 10°C and 45°C.

3. The authors explicitly differentiate this organism from those in the genus *Lactococcus* by positive growth not only at 45°C, but also in 6.5% NaCl and at pH 9.6 (Schleifer & Kilpper-Bälz, 1984, 1987; Schleifer et al., 1985).

4. *Ent. seriolicida* is α-haemolytic on BHI agar supplemented with sheep, horse and rabbit blood.

5. DNA reassociation analysis indicates *Ent. seriolicida* is most closely related to *Ent. hirae* of those enterococci used in the analysis (reassociation of 24%, relative to an homologous reassociation of 100%). No non-*Enterococcus* species were compared in the analysis, apart from *Escherichia coli.*

Pathogenicity

Symptoms occurring in fish with *Ent. seriolicida*-induced septicaemias include exophthalmia, haemorrhagic congestion of the internal organs (liver, spleen, kidney and intestines), petechia internal to the opercula, and accumulation of fluid at the bases of the pectoral and caudal fins. This syndrome is typical of the"streptococcicosis" first described among rainbow trout in Japan (Hoshina et al., 1958). Precise definition of the disease has been difficult because identity of the causative organisms has not been clarified.

Agents of "streptococcicosis"

An heterogeneity in the characteristics reported in the bacteria isolated from diseased fish suggests that "streptococcicosis" is a liberal term applicable to the similar pathological effects of several Gram-positive bacteria, probably themselves quite closely related. Despite a diversity of apparent serotypes (Austin & Austin, 1987), available serological information indicates that the disease is caused by members of the genus *Streptococcus,* in its former sense: that is bacteria of either the genus *Streptococcus, Enterococcus* or *Lactococcus* as currently defined (Garvie et al., 1981; Ludwig et al., 1985; Schleifer & Kilpper-Bälz, 1984, 1987; Devriese et al., 1993). In a limited numerical taxonomic study (Austin & Austin, 1987) two clusters of pathogens were described. The first cluster is heterogeneous with regard to haemolytic reaction, with organisms displaying either α-haemolysis, β-haemolysis or no haemolysis at all. The second cluster contains exclusively α-haemolytic strains which are characteristically negative for Lancefield group D antigen.

Streptococcicosis has been reported in the south-eastern states of Australia (New South Wales, Victoria and Tasmania) and was responsible for $6 million losses to the Tasmanian salmonid industry over the period 1983-91 (Carson, 1990).

Host range

Streptococcicosis has been reported in rainbow trout, *Oncorhynchus mykiss*, in Japan, South Africa, Australia and Italy (Hoshina et al., 1958; Carson et al., 1993; Bragg & Broere, 1986; Ceschia et al., 1992); yellowtail *Seriola quinqueradiata*, eels *Anguilla japonica*, ayu *Plecoglossus altivelis* and tilapia *Tilapia nilotica* in Japan; Atlantic croaker *Micropogon undulatus*, channel catfish *Ictalurus punctatus*, golden shiner *Notemigonous chrysoleuca*, hardhead catfish *Arius felis*, menhaden *Brevoortia patronus*, pinfish *Lagodon rhomboides*, silver trout *Cynoscion nothus*, spot *Leiostomus xanthurus*, stingray *Dasyatis* sp. and striped mullet *Mugil cephalus* in the USA (Austin & Austin, 1987, and refs. therein) and turbot *Scophthalmus maximus* in Spain (Toranzo et al., 1994). The disease has also been reported from Malaysia, Israel, the United Kingdom and Hungary (Carson, 1990). It occurs in both wild and farmed populations.

2.3.2 Genus *Enterococcus*

History

The genus *Streptococcus* (Rosenbach 1844) originally accommodated all catalase-negative, facultatively anaerobic and some strictly anaerobic gram-positive cocci (Hardie, 1986). Molecular and chemotaxonomic analysis of the constituent species has recently led to a comprehensive revision of the genus (Schleifer & Kilpper-Bälz, 1984; Schleifer et al., 1985). Three clusters of species become apparent under 16S rRNA catalogue analysis (Ludwig et al., 1985) and nucleic acid hybridisation analysis (Garvie & Farrow, 1981; Kilpper-Bälz & Schleifer, 1984; Schleifer & Kilpper-Bälz, 1984; Schleifer et al., 1985). This clustering is in essential agreement with earlier numerical taxonomic studies of the streptococci (Jones, 1978; Bridge & Sneath, 1983). Each cluster is now recognised as a separate genus:

* *Streptococcus*, comprising the bulk of the streptococci including the pyogenic and oral forms.
* *Enterococcus*, containing the enterococcal streptococci, sometimes described as the enterococcal Lancefield group D streptococci (Schleifer & Kilpper-Bälz, 1984).
* *Lactococcus*, containing the lactic acid streptococci, also described as the non-motile Lancefield group N streptococci (Schleifer et al., 1985).

Inter-generic differentiation

The streptococci have been distinguished on several classical criteria:

* *serological (Lancefield) groupings*

Originally, both formal and informal distinctions within the Gram-positive cocci referred to Lancefield antigen groups (Lancefield, 1933). Although the streptococci proper had no specifically definitive antigens, the enterococci were taken to be positive for group D antigen and the lactococci positive for group N antigen. Exceptions to both these generic markers occur, however. Antigenic determinants have many failings as criteria for primary classification. Many Gram-positive cocci have no group-specific antigen. Many conspecific strains differ markedly in the antigens they possess (Schleifer & Kilpper-Bälz, 1987). Few antigens are now considered to define exclusively a species or genus.

* *G+C content of genomic DNA*

All three genera have similar ranges: *Streptococcus* 35-46 mol%; *Enterococcus* 38-41 mol%; *Lactococcus* 34-38 mol% (with *L. raffinolactis* 40-43 mol%).

* *haemolysis*

Streptococcus and *Enterococcus* exhibit α-haemolysis, β-haemolysis or none (Schleifer & Kilpper-Bälz, 1987); *Lactococcus* species are usually non-haemolytic, though some strains of *L. lactis* have shown weak α-haemolysis (Schleifer & Kilpper-Bälz, 1987). Haemolytic reaction is therefore not a particularly useful diagnostic concept at the level of genera.

* *respiratory quinone comparisons*

L. lactis and *L. garvieae* use menaquinone 9 (MK-9) as their primary respiratory quinone. *L. lactis* subsp. *hordniae* alone of the lactococci uses MK-8. The other two *Lactococcus* species have no isoprenoid quinones. The enterococci use either menaquinones MK-7, MK-8 or dimethylmenaquinones DMK-8, DMK-9. However, many enterococci also lack isoprenoid quinones. No isoprenoid quinones have been detected in the *Streptococcus* species (Collins & Jones, 1979; 1981). Because so many species of these genera have no quinone content at all and MK-8, at least, is found across genus boundaries, the use of this trait for genus differentiation is limited.

Genus description

The generic description of the genus *Enterococcus* was based initially upon the phenotypic characters of the species *Ent. faecalis* and *Ent. faecium* that distinguished them from the non-enterococcal streptococci (Schleifer & Kilpper-Bälz, 1984, 1987). These include:

* growth at both 10°C and 45°C
* growth in 6.5% NaCl
* growth at pH 9.6
* Lancefield group D antigen
* pyrrolidonylarylamidase (PYRA)

The genus has since expanded to include a possible 19 species that share appropriate 16S rRNA sequence similarities (Devriese et al., 1993). Many of these recently assigned species do not, however, share all the definitive traits attributed to the genus in the past. Exceptions to each of the above criteria occur in the genus *Enterococcus.* Furthermore, many of these characters are found in species of the closely related genera *Streptococcus, Lactococcus, Aerococcus, Leuconostoc* and *Pediococcus* (Devriese et al., 1983; Schleifer et al., 1985; Facklam et al., 1989; Facklam & Collins, 1989; Martinez-Murcia & Collins, 1991; Elliott et al., 1991).

There are, in fact, no phenotypic criteria by which the genus *Enterococcus,* as it is currently constituted, can be unequivocally distinguished from the other gram-positive, catalase-negative cocci (Devriese et al., 1993).

2.3.3 *Enterococcus seriolicida* in Australia

An "enterococcal" bacterial strain was isolated from rainbow trout (*Oncorhynchus mykiss*) in Australia (Tasmania and Victoria) and South Africa over a two year period in the early 1990s (Carson et al., 1993). All strains from both countries showed very similar growth and biochemical profiles, with exceptions only in acetoin production and acid production from mannitol and mannose. This homogeneity of strains was confirmed by whole-cell protein profiles. Noteworthy traits of these strains include:

1. growth at 6.5% NaCl, but only after 48 hours, or 72 hours for some strains.
2. α-haemolysis on sheep's blood
3. Lancefield group N antigen negative; also contains no group D antigen
4. no growth at 45°C (after 24 hours - J. Carson, pers. comm.).

The phenotypic profile of the endemic "enterococcus" does not conform in all points with the profile published to define the *Ent. seriolicida* type strain (Kusuda et al., 1991). However, when phenotypes of both strains were examined in the same laboratory (Carson et al., 1993; Schmidtke & Carson, 1994), identical profiles were obtained. This prompts the proposition that the Australian "enterococcus" is *Ent. seriolicida.*

Comparable enterococcal pathogens

Report of diseased rainbow trout in South Africa (Bragg & Broere, 1986) parallels the description of the Australian/South African strains except insofar as the organism involved did not grow in 6.0% NaCl. However, as noted by Carson et al. (1993), the Australian/South African isolates grew in 6.5% salt only after 48-72 hours. Differences in experimental technique could explain this discrepancy.

Another "streptococcal" organism isolated from rainbow trout in Italy (Ceschia et al., 1992) is also comparable to the Australian/South African strains in its biochemical and growth properties. In these three instances, the pathogen phenotypes did not fall clearly within strict generic definitions, engendering a certain hesitation in the authors in assigning the pathogen to either the genus *Enterococcus* or *Lactococcus.*

In 1994 outbreaks of streptococcicosis were reported in farmed turbot populations in north-west Spain (Toranzo et al.). Because of an earlier reference to a close relationship between *Ent. seriolicida* and *L. garvieae* (Devriese et al., 1993 and citations therein), workers used both these species as reference organisms in their analysis. The biochemical and growth characters of the new isolate are identical with those of *L. garvieae*. They differ, however, from *Ent. seriolicida* in that there is no growth at 45°C and only poor, delayed growth in NaCl 6.5% and at pH 9.6. By comparison, the Australian isolate shows the characteristics of *L. garvieae* and the Spanish pathogen: no growth at 45°C and poor growth at 6.5% NaCl and pH 9.6.

2.3.4 Identity with *Lactococcus garvieae*

Ent. seriolicida was first described in 1991 (Kusuda et al.). In 1993 it was suggested (Devriese et al., 1993) that this species was synonymous with *L. garvieae*, a bacterium originally found in association with bovine mastitis and subsequently in human clinical samples (Garvie et al., 1981; Elliott et al., 1991). The basis for this identity was phenotypic similarity, 100% 16S rRNA identity (M. D. Collins, unpublished data, cited in Devriese et al., 1993) and protein analysis (Pot & Devriese, unpublished data, cited in Devriese et al., 1993).

There occurred in central Spain in 1991 a pathogen inducing classic enterococcal septicaemia in rainbow trout. The bacterium was analysed in tandem with the type species of both *Ent seriolicida* and *L. garvieae* (Domenech et al., 1993). Biochemical profiles were effectively identical; protein profiles showed no differences; and the (unpublished) sequence of the 16S rRNA gene of *Ent. seriolicida* was indistinguishable from the published sequence for *L. garvieae.*

Interestingly, Domenech et al. (1993) also reported both *L. garvieae* and *Ent. seriolicida* to be β-haemolytic, contrary to previous studies which showed *L. garvieae* to be non-haemolytic (Collins et al., 1983) or α-haemolytic (Toranzo et al., 1994) and *Ent. seriolicida* to be α-haemolytic (Kusuda et al., 1991; Toranzo et al., 1994). This study also failed to assess growth (or otherwise) at 45°C of the strains under consideration. This is an unfortunate oversight insofar as it is the principal phenotypic trait

that distinguishes the lactococci from the enterococci (Collins et al., 1989; Elliott et al., 1991; Devriese et al., 1993) and is pivotal to the confusion over the proposed species synonymy.

2.3.5 Genus *Lactococcus*

Genus Description

The non-motile, group N lactic streptococci have been recognised as a coherent group within the streptococci for some time (Jones, 1978; Garvie et al, 1981; Garvie & Farrow, 1981; Jarvis & Jarvis, 1981; Bridges & Sneath, 1982; Kilpper-Bälz et al., 1982). The genus *Lactococcus* was proposed formally in 1985 by Schleifer et al. on the basis of nucleic acid hybridisation data, immunological analysis using superoxide dismutase, similarities in lipotechoic acid structures, and comparable constituent lipids, fatty acids and menaquinones. These common attributes distinguished the lactic streptococci from those assigned to the genera *Streptococcus* and *Enterococcus.*

The attributes of the genus *Lactococcus* that distinguish it from the other two streptococcal genera are the ability of species to grow at 10°C but not at 45°C. Most strains are positive for Lancefield group N antigen. The lactococci are explicitly non-β-haemolytic: usually non-haemolytic but with rare instances of α-haemolysis in strains of *L. lactis* (Schleifer & Kilpper-Bälz, 1987). DNA has a G+C content of 34-43 mol%.

Taxonomy

The genus *Lactococcus* contains four species: *L. lactis* (with subspecies *lactis, cremoris* and *hordniae*), *L. garvieae, L. raffinolactis* and *L. plantarum.* (A fifth species, *L. piscium,* was proposed in 1990 by Williams et al.). Taxonomic studies on the genus are remarkably coherent (Schleifer et al., 1985). Immunological data, lipid and fatty acid analyses, respiratory quinones and lipotechoic acid structures consistently demonstrate a closely related group of species (Collins et al., 1983; Schleifer et al., 1985). This is confirmed by DNA-rRNA hybridisation (Garvie & Farrow, 1981) and DNA-DNA hybridisation studies (Garvie et al., 1981; Jarvis & Jarvis, 1981; Kilpper-Bälz et al., 1982; Schleifer et al., 1985). Genomic DNA reassociation values (cited as "homology values") of 15-25%

between even the most distantly related of the lactococci indicate very high levels of genetic relatedness within the genus (Schleifer et al., 1985). These data also show *Lactococcus* as genetically distinct from the other streptococci. 16S rRNA sequence comparisons (Collins et al., 1989; Williams et al., 1990) reaffirm the findings of hybridisation and chemotaxonomic work. *L. garvieae* and *L. lactis* (95% homology in 16S rRNA sequences), and *L. raffinolactis* and *L. plantarum* (96.5% homology) form two intrageneric lines by molecular, chemotaxonomic and physiological parameters.

2.3.6 *Lactococcus garvieae*

L. garvieae (NCDO 2155^T) was first isolated from bovine mastitis (Garvie et al, 1981). The species description, as *Streptococcus garvieae*, in 1981 (Collins et al., 1983) remarks the unusual cell wall peptidoglycan (lys-ala-gly-ala) which typifies the species; a preponderance of the menaquinone MK-9 and major fatty acids $C_{16:0}$ and $C_{18:1}$. Colonies are non-haemolytic. There is growth at 10°C, but none at 45°C. The species is positive for the serogroup N antigen. Uniquely among the lactococci, *L. garvieae* is PYRA positive, a trait usually characteristic of the enterococci (Facklam & Washington, 1991).

In 1991, Elliott et al. identified by molecular methods *L. garvieae* isolated from human clinical samples, some of which were associated with bacterial endocarditis. These strains had previously been misidentified as enterococci by classical methods (bile-esculin hydrolysis, growth at 6.5% NaCl, PYRA positive).

The sequence of the 16S rRNA gene of *L. garvieae* was published by Collins et al. in 1989. The sequence for the 16S rRNA gene of the type species of *Ent. seriolicida* was established as part of this project. Comparison of these two sequences is made here to confirm the identity of the two species, or otherwise establish their relationship.

3. METHODS

Reagents and stock solutions are described in Appendix I.

3.1 Bacterial Cultures

3.1.1. *Paracoccus halodenitrificans*

The type strain of *P. halodenitrificans* ATCC 13511^T (= DSM 735^T) was obtained from the American Type Culture Collection, Maryland, USA, Dec. 1991. The culture was so named and numbered on the acquisition form accompanying the culture. However, according to Urakami et al. (1990), the original strain ATCC 13511^T was lost and replaced with ATCC 25843, which was shown in 1990 to be something other than *P. halodenitrificans.* This culture was subsequently discarded and replaced with DSM 735^T. It seems unusual that the culture we received from the ATCC, subsequent to this replacement, bore the number of the original but no longer extant type strain. However, with no knowledge of the numerative protocols of the ATCC, and in deference to the reputation of the Collection, the organism received is presumed to be the type strain of *P. halodenitrificans.*

Cultures were grown in nutrient broth (Difco) supplemented with 6% NaCl at 25°C, and harvested after approx. 24 hours.

3.1.2 *Aeromonas* sp.

This bacterium (strain number 93/1061) was isolated from skin lesions of greenback flounder *Rhombosolea tapirina* at a fish farm in the north of Tasmania in 1993, by Dr. J. Carson. Cultures were maintained on Columbian Horse Blood (CHB) agar plates (Difco) at 25°C. Inoculants into brain-heart infusion broth (Difco) were shaken at 25°C and harvested when turbid.

3.1.3 *Enterococcus seriolicida*

The *Ent. seriolicida* type strain, ATCC 49156^T, was provided by Dr. Jeremy Carson (Fish Health Unit, Department of Primary Industries and Fisheries, Launceston, Tasmania). Cells were cultured in 250 ml Todd-Hewitt broth supplemented with 0.2% DL-threonine, at 25°C. Prior to DNA extraction,

cultures were treated with peptidoglycan-directed antibiotics during the final replicative cycle as described below.

3.2. DNA Extractions

3.2.1 DNA extraction (*P. halodenitrificans, A. salmonicida*)

DNA was extracted from bacterial cultures by a method modified from that of Marmur (1961).

cell lysis

Pelleted cells from a 250-500 ml culture were resuspended in 8-10 ml saline EDTA. Lysozyme was added to a final concentration of 2 mg/ml, and the suspension was incubated for 1 hour at 60°C. Cells were checked microscopically to confirm lysis.

deproteination

Sodium dodecylsulphate (SDS) was added to the lysate to a final concentration of 1% to advance cell lysis and inhibit nuclease activity. 1ml proteinase K (10 mg/ml) was added and the lysate incubated at 37°C for a further 15 min. 0.25 volumes of 70% sodium perchlorate was added to accelerate the dissociation of proteins from nucleic acids, and the lysate was shaken for a further 15 min.

extraction

The mixture was extracted with phenol-chloroform-isoamyl alcohol (24:24:1) once and then again with chloroform-isoamyl alcohol (24:1). (Isoamyl alcohol reduces foaming). Phase separation by centrifugation allowed removal of nucleic acids with the aqueous layer from above the denatured proteins and cell debris collected in the white flocculent at the phase interface. DNA and RNA were spooled with approx. two volumes of ice cold absolute ethanol and air-dried.

Spooled nucleic acids were dissolved in milli-Q water. RNAse A (10 mg/ml) was added to a final concentration of 40 μg/ml and incubated for 30 min at 37°C. DNA was re-precipitated with ethanol, dried and dissolved in milli-Q

H_2O. It was examined for integrity and purity on a 0.8% agarose/TBE gel (Butterworth, 1976) containing ethidium bromide (final concentration 0.5 μg/ml) at 8-10 V/cm for approx. 1 hour.

3.2.2 DNA extraction from *Ent. seriolicida*

Ent. seriolicida proved refractory to lysis to a degree beyond expectation or experience. Freeze-thaw, sonication and hand-operated french-pressure cell failed to disrupt the cells. The Marmur method was modified to incorporate pre-treatment with an antibiotic during the final growth cycle (as recommended by Dr. Jeremy Carson) to weaken cell walls and hasten lysis. The concentration of lysozyme was increased and the period for its activity extended.

Cells were cultured in broth as described above. After 14 hours, penicillin G (benzyl penicillin) was added to a concentration of 0.3 g/250 ml culture and vancomycin to a concentration of 12.5 mg/250 ml culture. Cells were grown for a further 1 hour, then harvested.

Pelleted cells were resuspended in saline EDTA, with lysozyme to a final concentration of 10 mg/ml, and incubated for 36 hours at 60°C. SDS was added to a final concentration of 2%, with 1 ml proteinase K (10 mg/ml), and incubated at 37°C for a further hour. Cells were checked microscopically for lysis, and then extracted as described above.

3.2.3 DNA quantification

DNA was quantified by A_{260} absorbance, using the relationship:

$$[\text{DNA } \mu g/ml] = 50 \times A_{260} \times \text{dilution factor}$$

which is derived from the datum that double-stranded DNA has an A_{260} of 1.0 at a concentration of 50 μg/ml (Sambrook et al., 1989). DNA was diluted to approx. 1.0 μg/μl.

3.3 Amplification of 16S rDNA

The bacterial 16S rRNA gene was selectively amplified by polymerase chain reaction (Saiki et al., 1985). Highly conserved sequences at each end of the gene were used as priming sites for synthetic oligonucleotides, primer A and primer H, as described by Edwards et al. (1989) (Table 3.1).

Primer A binds the antisense, coding strand of the gene at position 8-27 and primer H binds the sense, non-coding strand at position 1541-1522 (*E. coli* numbering, [Neefs et al., 1993]). These primers facilitate the amplification of a fragment of approx. 1500 nucleotides, encompassing almost the entire gene.

PCR was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus) using reagents from Perkin Elmer Cetus unless otherwise specified. A standard 100µl reaction contained 10 ng genomic DNA as template, 50 pmol primers A and H, 10 µl 10x PCR buffer II (500 mM KCl, 100 mM Tris-HCl, pH8.3), 200 µM each dNTP (total 0.8 mM dNTPs) (USB), $MgCl_2$ 1.0 mM, 2.5 units *AmpliTaq* ®DNA polymerase and milli-Q water to 100µl. Reactions were covered with 80 µl sterile mineral oil. Ten replicate reactions (=1 ml in total) were regularly done simultaneously to generate template for subsequent sequencing reactions.

The PCR was performed over 35 cycles. Each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min and extension for 6 min at 72°C (Edwards et al., 1989). Although this 6 minute extension time seemed excessive and had every indication of being a misprint in the original paper, reducing the extension time to more conservative 2 or 3 minutes significantly reduced the yield of PCR product. There is precedence for the use of an extension time of 6 minutes in the amplification of 16S ribosomal genes by PCR (Sogin, 1990).The phenomenon of PCR primers binding non-specifically that has been correlated with protracted extension times (Saiki et al., 1988) was not observed.

An annealing temperature of 55°C is a compromise between the specificity achieved at high annealing temperatures and the greater yield at lower temperatures. Non-specific binding is reduced and reaction sensitivity improved at this temperature, compared to earlier PCR with thermostable polymerases where annealing temperatures of 37-40°C were used (Saiki et al., 1988).

While the standard recipe was unfailingly successful at the outset, results became erratic over time (as they did for everyone in our research group). Rigorous investigations into possible causes for this were fruitless (these included monitoring temperature profiles of the thermocycler, use

of different thermocyclers and cycling regimes, different laboratories, change of polymerases, many different primer couples and milli-Q water from different sources). PCR was only successfully and predictably re-established with creative departures from theoretically "correct" reaction conditions. Each new template, priming pair of oligonucleotides and each new batch of Taq polymerase demanded an assay for optimal concentrations of constituents. Optimal concentrations of template ranged from 10 ng to 2 μg, and of primers from 50 pmol to 1 μg. The template/primer ratios varied unsystematically during optimisation. Variation in other reaction components, including $MgCl_2$, had little effect.

During the period when the standard PCR was no longer reliable, two alternative cycling protocols were tried. The first attempted was "hot start" (Erlich et al., 1991): primers are added to the reaction after an initial denaturation step at 94°C for 3 min. The second method tried was the "touchdown" program (Don et al., 1991): the annealing temperature is set well above the melting temperature of the primer/template duplices in the early cycles and drops progressively through the run until it reaches the predicted T_m - 5°C for the final 20-30 cycles. Other temperature parameters remain unchanged. These protocols are both designed to minimise non-specific priming. However, neither alternative contributed to the solution of the PCR problems.

As sequencing of the 16S rDNA progressed, shorter fragments encompassing unsequenced regions were amplified in the same manner using primers internal to the gene. Amplification of shorter fragments gave greater yields than longer fragments and also proved an economical use of primers. In these instances, pairs of sequencing primers were chosen (see Table 3.1, fig 3.1) which primed opposite strands in such a way as to polymerise convergently, amplifying the intervening sequence.

3.4 Purification of PCR Product

Products of the PCR were run against low molecular weight markers (Amresco) on 1% agarose/TAE gels containing ethidium bromide (0.5 μg/ml) at 8-10 V/cm for 30 min to 1 hour. DNA was visualised with UV transillumination, and the ~1500bp fragment excised with a scalpel. DNA was removed from the agarose and purified using the GENCLEAN II® Kit

(Bio 101 Inc., La Jolla, CA, USA) according to manufacturers instructions. Yield of PCR product was estimated from A_{260} measurements (as above).

Recoveries were inevitably low. A comparable 100 µl reaction containing 0.8 mM dNTPs contains sufficient precursor to yield 25 µg of amplified product (Saiki, 1989; Innes & Gelfand, 1990; Steffan & Atlas, 1991). This is at least an order of magnitude greater than anything achieved in our laboratory using the protocol and its permutations described here. The intensity of fluorescence of the pre-purified PCR product when visualised with ethidium bromide under UV illumination suggested that the PCR itself generates very much more DNA than is finally recovered. It would seem that most loss occurs during purification. To overcome this problem, various alternatives to Genclean II® were tried. Although yields improved, degeneration in the quality of the sequence subsequently recovered prompted a return to the Genclean II® method.

3.5 Sequencing Amplified 16S rDNA

Dideoxynucleotide chain termination sequencing (Sanger et al., 1977) adapted for double stranded PCR product (Bachmann et al., 1990), and further modified by S. Dobson (PhD thesis, 1993), was used to sequence this major segment of the 16S rRNA gene. There is an inherent problem in sequencing relatively short linear fragments of DNA because the tendency of template molecules to reanneal to one another largely precludes the annealing of primer molecules to template strands. This method is noteworthy insofar as it overcomes this problem in two ways: the detergent nonidet P40 is added at the annealing step and the annealing process itself involves boiling and snap-freezing the primer/template mix.

The Sequenase® Version 2.0 Kit (USB) was used throughout, including Sequenase®T_7 DNA polymerase. Fragments were labelled with α-^{35}S-dATP (12.5 mCi/ml) or α-^{33}P-dATP (10 mCi/ml).

3.5.1 Sequencing Primers

Sequencing primers are described in Table 3.1. Priming locations within the prokaryote 16S rRNA molecule are numbered according to Neefs et al.

(1993). Those taken directly from the publications cited were designed to bind presumed invariant regions of the 16S rDNA as established by examination of a large set of known rDNA sequences. These include primers A and H used for amplification of the fragment. Some primers (specified *) were modified from the original reference for previous purposes in the laboratory (modifications in bold).

P. halodenitrificans was sequenced using primers A, H, 19, 23, WS8, 26, 27, 20, 21, WS5. Single base mismatches occur between the bacterial 16S rRNA sequence and primers 20, 21, 23 and WS5.

A. salmonicida was sequenced using primers A, H, 20, 26, WS5, 27, 19, 23, WS6 and 21. A single base mismatch occurs between 16S rRNA sequence and primers 21 and WS5. There is a three base mismatch between the 16S rDNA sequence and the 5' end of primer WS6.

Ent. seriolicida was sequenced using primer A, H, WS5, 19, 27, 21, 23 and WS8. Primer WS5 has a one base mismatch relative to the 16S rRNA sequence.

Primers were used in a such a way that every part of the gene fragment was sequenced at least twice from one or both strands (Fig. 3.1), and any ambiguous sequence was re-sequenced until the ambiguity was resolved. Where this was not possible, an "n" was specified in place of a base in the sequence published.

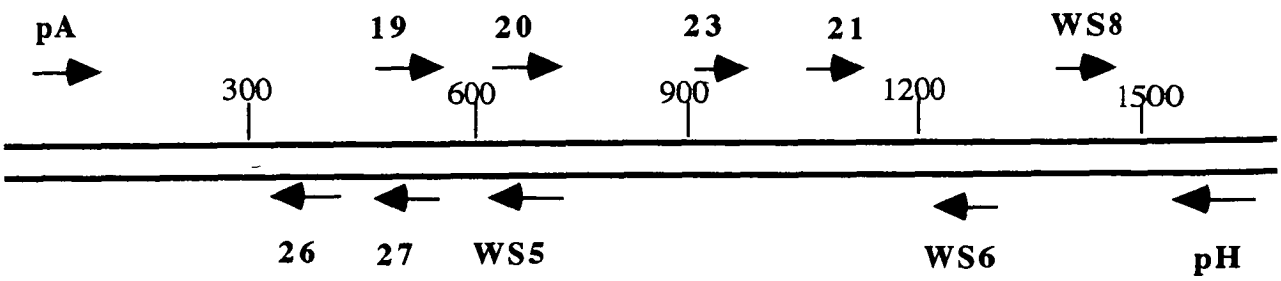

Fig. 3.1: Target sites on the 16S rRNA gene for sequencing primers

Primer	Location	Sequence	Reference
primer A	8-27	AGAGTTTGATCCTGGCTCAG	Edwards et. al. 1989
primer 26	360-341	CCCACTGCTGCCTCCCGTAG	Edwards et. al. 1989
primer 19	519-536	CAGCAGCCGCGGTAATAC	Edwards et. al. 1989
primer 27	536-519	GTATTACCGCGGCTGCTG	Edwards et. al. 1989
primer 20	685-704	GTAGCGGTGAAATGCGTAGA	Embley et. al. 1988
WS5*	704-685	TCTATGCATTTCACCGCTAC	Embley et. al. 1988
primer 23	907-926	AAACTCAAAGGAATTGACGG	Edwards et. al. 1989
primer 21	1100-1115	CAACGAGCGCAACCCT	Embley et. al. 1988
WS6*	1245-1226	CTACCATTGTAGCACGTGTG	Stackebrandt & Charfreitag, 1990
WS8 1988	1392-1406	GTACACACCGCCCGT	Embley et. al.
primer H	1541-1522	AAGGAGGTGATCCAGCCGCA	Edwards et. al. 1989

Table 3.1: Oligodeoxynucleotide primers used for sequencing and PCR; *E. coli* numbering (5' → 3') in accord with Neefs et al., 1993.

Synthetic oligonucleotide primers were purchased from Bresatec Ltd., de-salted but containing fore-shortened failure sequences (Gait, 1984; Brown & Brown, 1992)). Longstanding laboratory stocks had been purified previously to homogeneous full-length oligonucleotides. As new stocks were purchased, however, this purification was abandoned and oligonucleotides were used as heterogeneous mixtures ranging from full-length to 3-4 bases fore-shortened. Although HPLC analysis indicated that some of the Bresatec oligonucleotides were of poor quality, with a large proportion of failure sequences present (up to 80%), the presence of these foreshortened primers did not seem to affect the fidelity of the

replication. Mismatches within the primers also had no effect on the efficiency of the sequencing reactions. Indeed, the sequence obtained when primer WS6 was used with the *Aeromonas* species was clear, unambiguous and correctly placed despite a three-base mismatch between the primer and the priming site. These observations suggest that the priming sites used are so definitive relative to the sequence of the rest of the gene that a certain amount of wobble is tolerated. A three base mismatch at the 5' end of a primer is less likely to affect polymerisation, which occurs at the 3' end of the primer.

3.5.2 Sequencing reactions

Annealing Reaction

The following reagents were combined in a microcentrifuge tube, boiled for 3 min and immediately snap-frozen in liquid nitrogen.

Sequencing primer (10 pmol/μl)	-	1.0μl
DNA template (1μg/μl)	-	1.0μl
5x reaction buffer	-	2.0μl
Nonidet P40 (20%)	-	1.0μl
dH_2O	-	5.0μl

Tubes were spun briefly to collect condensate and then placed on ice.

Labelling Reaction

Sequenase®T7 polymerase was diluted 1:8 in "enzyme dilution buffer"; or inadvertently in dH_2O with no ill effect.

To the 10μl annealed reactants were added in the following order:

0.1M dithiothreitol	-	1μl
Labelling mix (dGTP), undiluted	-	2μl
α-^{35}S-dATP (12.5 mCi/ml)	-	1.5μl
T7 DNA polymerase (1:8)	-	2μl

The reaction proceeds at room temperature over times ranging from 3 min for sequence close to the primer, to 5 min for sequence further from the primer. When sequence very close to the primer was sought, 1μl Mn^{2+} buffer from the Sequenase 2.0® kit was added to the labelling reaction, sometimes with good effect and sometimes with negligible.

α-^{33}P-dATP(10mCi/ml) was substituted periodically for α-^{35}S-dATP, initially to evaluate its claim to increased sensitivity in dideoxytermination sequencing (Evans & Read, 1992). This claim is justified by a maximum emission energy (0.249 MeV) 50% greater than that of the ^{35}S isotope (0.167 MeV). My experience was that the resolution achieved by each analogue is indistinguishable. The phosphate isotope has the disadvantage of a considerably shorter half-life (25.4 days for ^{33}P, 87 days for ^{35}S), diminishing storage times for reactions that may require a second electrophoretic separation. However, the choice of isotope was finally based on cost (^{33}P) and availability (^{35}S), or ultimately what happened to be in the frig. on the day.

Termination Reactions

The labelling reaction was terminated by removing 25% of the reaction mix into one of four tubes each containing a dideoxynucleotide corresponding to one of the four bases i.e. ddATP, ddCTP, ddGTP and ddTTP. In this way are the polymerising fragments in each tube terminated at a preordained base. The four termination mixes (2.5µl) were pre-warmed to 37°C, 3.5µl of the labelling reaction added and the reactions incubated for three minutes at 37°C.

The termination reaction was stopped by the addition of 4µl formamide-based STOP solution. Reactions were stored at -20°C for subsequent use, though the addition of Mn^{2+} buffer in the labelling reaction shortens storage time to 2-3 days (USB recommendation).

dGTP/dITP Reactions

A second set of reactions was performed simultaneously using the dGTP analogue, dITP (deoxyinosine 5'-triphosphate) and the labelling mix and termination mixes specific to its use (Sequenase 2.0® kit - USB). When the sequence of a DNA fragment confers a strong tendency to secondary structure, it is not always fully denatured when run on denaturing PAGE. This causes fragments to run irregularly, giving rise to the phenomenon of band compression. Compression is a gel artefact. dITP is recommended for sequence with this tendency because the inosine analogue forms weaker hydrogen bonds than its guanosine counterpart, and thus disrupts the secondary structure that gives rise to band compression. However,

during the polymerisation reaction the action of the polymerase enzyme may be interrupted by strong secondary structure of the template and this seems to be more pronounced when dITP is used than dGTP. This phenomenon causes cross-banding on the sequencing gel as the polymerisation "pauses" at the same place on the template non-base-specifically. Cross-banding is a polymerisation artefact and is best addressed by use of dGTP. Use of both analogues maximises the likelihood of well resolved sequence (Step by step protocols for DNA sequencing with Sequenase® Version 2.0, 5th edition, USB and references therein).

3.5.3 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed using the LKB Macrophor Sequencing System (Pharmacia LKB), including the LKB 2010-001 Macrophor apparatus and the LKB Multitemp II Thermostatic Circulator. Cleaned plates were treated with Bind-Silane and Repel-Silane (Pharmacia LKB) as recommended by the manufacturer.

Reaction products were separated on a 0.2mm 6% denaturing (7M urea) polyacrylamide gel in 1xTBE buffer, at 1800V, with water circulating through the thermostatic plate at 55°C. Before loading, the samples were denatured at 90°C for 3 min.

Four dGTP reactions (terminating at A, C, G and T) were run immediately adjacent to the four dITP reactions derived from the same primer. Equivalent reactions were run for 2 hours and 5 hours side by side; these sequences overlap and gave a combined range of up to 200-300 bases of readable sequence per primer.

Gels were fixed in 10% acetic acid (v/v) for 20 min while still attached to the non-thermostatic sequencing plate. Gels were air-dried, then exposed to XOMAT-AR film (Eastman Kodak Co.). Autoradiographs were usually developed after 24 hours, though if the radiation counts from the gel were high, exposure times could be as short as 8-12 hours with no obvious loss of resolution.

3.6 Taxonomic Analysis

3.6.1 Sequence alignments

Sequences were read from autoradiographs and recorded using the program Seqspeak 1.0 DNA Sequence Editor by K. Conover (1991, Dalhousie University, Nova Scotia, Canada). Overlap in sequences derived from different primers was identified by eye. Ambiguous nucleotides were recorded as "n" in the sequence.

The taxonomic position of each bacterial species in this study was already strongly suggested by either phenotypic, chemotaxonomic and/or molecular evidence. Consequently, the 16S rDNA sequence of each species was aligned with the 16S rDNA of the species presumed on this evidence to be most closely related. In the case of both *P. halodenitrificans* and *Ent. seriolicida*, the type species of the genera to which each is currently assigned was also included in the alignment. Published sequences were taken in a pre-aligned form from the Ribosomal Database (RDB, Olsen *et al.*, 1991). The new sequence was added into the alignment manually using the HOMED (Homologous Sequence Editor) program (Peter Stockwell, 1986 University of Otago, NZ), accessed through ANGIS (Australian National Genomic Information Service - University of Sydney, NSW).

The 16S rRNA sequence of *P. halodenitrificans* was aligned against those of *P. denitrificans* LMG 4218^T, (EMBL accession number X69159, as cited by Ludwig et al., 1993) and members of the family Halomonadaceae: *Deleya marina* (DSM 4741^T), *D. halophila* (DSM 4770^T), *Halomonas elongata* (DSM 2851^T), *H. halmophila* (ATCC 19717^T), *H. subglaciescola* (ACAM 12^T), *D. aquamarina* (DSM 30161^T) and *Halovibrio variabilis* (DSM 3051^T), (GSDB accession numbers M93352-M93358 respectively). Sequences of bacteria from the γ-subclass of the Proteobacteria were taken from the Ribosomal Database and used as references: *Oceanospirillum linum* (ATCC 11336^T), *Pseudomonas aeruginosa* (ATCC 25330^T) and *Escherichia coli* (culture collection number unspecified).

The 16S rRNA gene sequence of the endemic strain of *A. salmonicida* was compared with the sequences of *A. salmonicida* subsp. *salmonicida* NCMB 1102^T, *A. salmonicida* subsp. *achromogenes* NCMB 1110^T and *A. salmonicida* subsp. *masoucida* NCMB 2020^T (EMBL accession numbers X60404, X60405,

and X60406, respectively). Other aeromonad 16S rRNA sequences extracted from the Ribosomal Database were added into the alignment were those of *A. hydrophila* ATCC 7966^T (=NCMB 9240^T), *A. trota* ATCC49657^T, and two *Aeromonas* species (RDB accession numbers AS9533 and AS9701). Insofar as the sequences showed 100% identity with several subspecies of *A. salmonicida*, more extensive comparisons were not made.

The 16S rRNA sequence of *Ent. seriolicida* was aligned with sequences taken pre-aligned from the RDB. These are *Ent. faecalis* (submitted to the RDB by the Woese laboratory, with no information regarding strain), *Ent. sulfureus* (NCDO 2379^T, GSDB accession number X55133), *L. lactis* subsp. *lactis* (strain 7962, Salama et al., 1991, GSDB accession number M54921), *L. lactis* subsp. *cremoris* (ATCC 19257, GSDB accession number M58836), *L. garvieae* (NCDO 2156, GSDB accession number X54262), *L. raffinolactis* (ATCC43920^T,NCDO617^T, GSDB accession number X54261), *L. piscium* (NCFB 2778^T, GSDB accession number X53905), *L. plantarum* (NCDO1869^T,ATCC 43199^T, GSDB accession number X54259), *Streptococcus bovis* (ATCC 33317, GSDB accession number M58835) and *E. coli* (Brosius et al., [1978], GSDB accession number J01695).

3.6.2 Distance Analysis

The PHYLIP v3.4 programs (Phylogeny Inference Package) (Felsenstein, 1989), accessed through ANGIS, were used to analyse the data from alignments. The DnaDist program calculated the Jukes-Cantor evolutionary distances from pairwise sequence differences. The program FITCH converted the distance data into evolutionary relationships among species using random order input of sequences and global rearrangements. These data were expressed as phylogenetic trees using the program DRAWTREE. Percentage similarity data were calculated using PAUP v3.0 (Swofford, 1991).

3.7. Probe Design

The alignments of 16S rRNA sequences used to establish the taxonomic position of each bacterial strain are described above. The alignments for *A. salmonicida* sp. and *E. seriolicida* were examined by eye for regions in which sequence for each bacterium departed maximally from that of its near relatives. These potential probe sites were then compared with

sequences listed in the RDB, and with those in the GSDB within the categories of phage, bacterial, structural RNAs and viral, to establish their general occurrence, and to identify sequences with highest similarity to them and the degree of that similarity. The FastA Program, which uses the Lipman-Pearson algorithm (Lipman & Pearson, 1985) and is accessed through ANGIS, was used to this purpose. Two probes were designed that would bind sequences unique to each bacterial strain in such a way that the region of sequence between them would be selectively amplified by a polymerase chain reaction.

4. RESULTS

E. coli numbering (Gutell, 1993) is used throughout the text below to describe sites on the 16S rRNA molecule and gene sequence. Helices on the rRNA molecule are numbered according to Neefs et al., 1993.

Sequences were lodged with the Genome Sequence Data Base (GSDB) under accession numbers:
Paracoccus halodenitrificans - L04942
Aeromonas species - L31915
Enterococcus seriolicida - L32813

4.1 *Paracoccus halodenitrificans*

The sequence for the 16S rRNA gene of *Paracoccus halodenitrificans* ATCC 13511^T is deposited with the Genome Sequence Data Base (GSDB) under the accession number L04942.

To clarify the taxonomic status of *P. halodenitrificans* the sequence of its 16S rRNA gene was compared with the 16S rRNA gene sequences of the type species *P. denitrificans* and of members of the family Halomonadaceae. Relationships between these species are described by the phylogenetic tree of Fig. 4.1. The percentage similarities between *P. halodenitrificans* and the compared species are shown in Table 4.1. These comparisons assign *P. halodenitrificans* to the family Halomonadaceae. The 16S rRNA sequence of *P. halodenitrificans* was examined for the 17 signatures which define the Halomonadaceae (Dobson et al., 1993). These signatures all occur in the sequence, including the cytosine at position 486 that distinguishes members of the family.

4.2 *Aeromonas salmonicida*

Aeromonas strain 93/1061 in northern Tasmania, was tentatively presumed to be a subspecies of the pathogen *Aeromonas salmonicida*. The sequence of the 16S rDNA of this strain has been lodged in the GSDB under the accession number L31915.

A. salmonicida strain 93/1061 was aligned with *A. hydrophila* (ATCC 7966) and *A. trota* (ATCC49657^T), and with several strains of *A. salmonicida* subsp. *salmonicida* including the type strain (NCIMB 1102^T), and subspecies *masoucida* (NCIMB 2020^T, EMBL accession number X60407) and *achromogenes* (NCIMB 1110^T, EMBL accession number X60406). The 16S rDNA sequences of the latter two subspecies are identical (Martinez-Murcia et al., 1992a). The 16S rRNA sequence of the flounder-derived *Aeromonas* strain 93/1061 has 100% identity with that of *A. salmonicida* subspp. *achromogenes* and *masoucida.*

4.2.2 PCR Primers:

Sites on the 16S rRNA gene chosen as targets for priming in PCR are:

As1. Sequence: 5'-TTTCGCGATTGGATGAA-3'; 17-mer; G+C=41%
Site: 216-232
Specificity: This site is identical for *A. salmonicida* subspp. *salmonicida, achromogenes* and *masoucida,* and also for *A. sobria* and *Aeromonas* sp. ATCC 49568; has a one base difference with *A. eucrenophila, A. jandaei, A. ichthiosmia, A. veronii* and *A. schubertii*; differs from *A. hydrophila, A. caviae, A. media* and *A. trota* by 4 bases. Beyond the aeromonads, the nearest consensus is a single base mismatch with *Coxiella burneti* citrate synthase gene (GSDB code CBNMRSA), with *Shewanella alga* 16S rRNA gene (GSDB SH16RRNAB) and the 16S rRNA sequences of various barophilic bacterial strains.

As2. Sequence: 5'-TTGGCGCCTATTACGTGTCAA-3'; 21-mer; G+C=50%
Site: 479-456
Specificity: species-specific. Within the aeromonads, the closest to consensus is the 3-base mismatch with *A. hydrophila. A. sobria , A eucrenophila* and *A. media* have 5 mismatches; *A. schubertii* has 6 mismatches; *A. trota, A. jandaei* and *A. caviae* have 11 mismatches. Beyond the aeromonads, the closest sequences have a 4 base mismatches. Three occur within the *Acinetobacter* sp. PHA synthase gene, within a region on a plasmid from *Ent. faecalis* and also within the *B. subtilis* sec A protein gene. There is a 5 base mismatch with a region of the *E. coli* chromosome.

- The target site for this primer is the site given by Dorsch et al. (1994) at which occur the signature sequences discriminating species of the genus *Aeromonas.* The primer does not distinguish *A. salmonicida* subspp.

salmonicida, achromogenes and *masoucida*. (Sequence for *A. salmonicida* subsp. *smithia* is unavailable.)

The product of a PCR using these two primers on genomic DNA from *A. salmonicida* strain 93/1061 is 261 base pairs in length. When a PCR was performed on genomic DNA extracted from a pure culture of *A. salmonicida* strain 93/1061 with primers As1 and As2, a fragment of this size was amplified. (These results are not shown as the reactions were not performed by me and the result therefore do not form part of this thesis.)

4.3 *Enterococcus seriolicida*

The sequence for the 16S rRNA gene of *Ent. seriolicida* ATCC 49156^T was lodged with the GSDB under the accession number L32813.

Recent publications have suggested that *Ent. seriolicida* and *L. garvieae* constitute a single species on the basis of identical 16S rRNA gene sequence and revised phenotypic characteristics (Domenech et. al., 1993; Devriese et al., 1993; Toranzo et al., 1994). 16S rRNA sequence data certainly suggest that *Ent. seriolicida* is better accommodated in the genus *Lactococcus* than with the enterococci. This is clearly indicated when the distance data are used to describe the phylogenetic tree of Fig. 4.2. The percentage similarities between *Ent. seriolicida, L. garvieae* and other compared species are shown in Table 4.2. Comparisons between the 16S rDNA sequence from *Ent. seriolicida* derived here and that for *L. garvieae* NCDO2156 deposited in the Ribosomal Database (GSDB accession number X54262) and also presented by Collins et al. (1989), show 7 base differences between the two. These differences are itemised below.

4.3.1 Sequence differences between *Ent. seriolicida* and *L. garvieae*

1. At position 191, all sequences in the alignment including *E. coli* and *Ent. seriolicida* have guanine, except *L. raffinolactis*, which has adenine. *L. garvieae* has uracil. This site is highly variable among prokaryotes (Neefs et al., 1993).

2. Within the 8 base insertion between *E. coli* positions 193 - 194, forming an extension to helix 10 (Neefs et al., 1993) which is common to the lactococci, streptococci and enterococci, *Ent. seriolicida* has C where *L.*

garvieae has G. The *Ent. seriolicida* sequence maintains canonical base-pairing across the helix; the *L. garvieae* sequence does not. There is no clear consensus at this position among the sequences compared, though base-pairing is maintained for all sequences except *L. garvieae.*

3. At position 194, all lactococci, streptococci and enterococci examined have G including *L. garvieae. E. coli* has cytosine. *Ent. seriolicida* has A. Because the adenine in the *Ent. seriolicida* sequence seems such an aberrant departure from the consensus at this position, this part of the gene was re-sequenced several times, though only from one direction. On each occasion did unambiguous sequence confirm the finding of an A at position 194. This sequence position is rated as highly variable over the prokaryotes (Neefs et al., 1993).

4. At position 1027, the sequence for *L. garvieae* shows a one-base deletion, in common with *L. raffinolactis,* relative to *E. coli* and the other sequences taken from the Ribosomal Database. The sequences of *E. coli,* the enterococci, *L. lactis* subspp. *lactis* and *cremoris* and *Ent. seriolicida* reported in the RDB have cytosine at this position. The sequence for *Lactococcus lactis* subsp. *lactis* reported in the literature (Collins et al., 1989), however, has a single base deletion at this site. There seems no general consensus at this position among the lactococci. *L. plantarum* and *L. piscium* have adenine. *S. bovis* has uracil. This sequence position is cited as a highly variable site over the prokaryotes (Neefs et al., 1993).

5, 6. At position 1091 and 1092, the published sequence of *L. garvieae* reads GG. All other sequences in the alignment have UA at these positions, including *Ent. seriolicida* and *E. coli.* According to the analyses of Neefs et al. (1993), uracil and adenine are highly invariant in these positions throughout the prokaryotes.

7. At position 1203, *L. garvieae* alone of the aligned sequences has guanine; other sequences have cytosine at this position. Cytosine is highly invariant at this position over the whole of the prokaryotes (Neefs et al., 1993)

Sequence similarities between *Ent. seriolicida* and *L. garvieae*

Sequence similarities between *Ent. seriolicida* and *L. garvieae* are described in full in Appendix 2.

4.3.2 PCR Primers:

Sites on the 16S rRNA gene chosen as targets for priming in PCR are:

Es1. Sequence: 5'-CGAGCGATGATTAAAGATAG-3'; 20-mer; G+C=40%

Site: between 63-72, within helix 6.

Specificity: species-specific. *Ent. seriolicida* 16S rRNA sequence in this region is quite different from that of other lactococci, even in length, due to heterogeneity in helix 6. Nearest consensus is 4 mismatches with *B. subtilis* chemotactic response protein, and 5 base mismatches with *S. aureus* DNA polymerase III gene and the Na^+ATPase α and β subunits of *Ent. hirae*. Of the RDB sequences, the closest approximation to the Es1 primer is a 5 base mismatch with *Prevotella orvis*.

2.) Sequence: 5'-ATAAGAATCATGCGATTCTCA-3'; 21-mer; G+C=30%

Site: 193-184, within the extension to helix 10 common to the lactococci, streptococci and enterococci.

Specificity: species-specific. Sequence at this site differs markedly from sequences of the other lactococci. The closest bacterial 16S rRNA gene is that of *Syntrophospora bryantii* with 5 mismatches. The NADP-dependent alcohol dehydrogenase gene of *Clostridium beijerinckii* has 4 mismatches with the Es2 primer, as does the Na+ATPase α and β subunit genes of *Ent. hirae*.

- This primer has two mismatches with the published *L. garvieae* sequence (differences 1 and 2), so the use of this primer is predicated on confidence in the sequence presented in this thesis.

The product of a PCR with these two primers on the genome of *Ent. seriolicida/ L. garvieae* is a fragment of 145 base pairs. When a PCR was performed on genomic DNA extracted from a pure culture of *Ent. seriolicida* with primers Es1 and Es2, a fragment of this size was amplified. (These results are not shown as the reactions were not performed by me and the result therefore do not form part of this thesis.)

		2	3	4	5	6	7	8	9	10	11	12
1	P.denitrif.	81.9	81.2	81.8	79.1	81.2	81.6	82.6	80.1	80.6	78.9	79.9
2	D.aquamar.	-	96.8	95.5	92.7	94.2	94.3	93.8	88.5	87.8	86.0	93.8
3	Hv.variab.		-	94.7	91.2	93.4	94.2	93.4	87.9	87.0	85.3	91.7
4	H.subglac.			-	93.5	95.0	94.8	93.4	88.2	86.9	85.4	93.7
5	H.halmo.				-	96.2	94.4	92.2	87.9	87.0	82.9	92.3
6	H.elongata					-	96.8	94.2	88.4	87.3	84.8	93.8
7	D.halophila						-	95.3	89.2	87.4	85.8	94.3
8	D.marina							-	89.4	87.5	86.0	93.5
9	O.linum								-	88.5	84.4	88.3
10	Ps.aerugin.									-	84.6	86.3
11	E.coli										-	84.6
12	P.halodenit.											-

Table 4.1

Percentage similarity between species of the genus *Paracoccus* and members of the family Halomondaceae. (Mean pairwise distances between taxa, with adjustment for missing data, calculated using PAUP v3.0.)

		1	2	3	4	5	6	7	8	9	10
1	E.coli	-									
2	Ent. faecalis	77.3	-								
3	Ent. sulfur.	77.2	94.6	-							
4	L.lactis lactis	76.8	86.7	86.4	-						
5	L. cremoris	76.8	86.5	86.3	99.4	-					
6	L. garvieae	77.6	86.3	86.6	92.5	93.0	-				
7	Ent. seriol.	77.4	88.4	88.2	94.2	94.2	99.4	-			
8	L. raffino.	77.1	87.8	89.0	92.0	92.0	92.0	94.5	-		
9	L. piscium	77.2	87.8	92.2	92.2	92.3	92.1	94.7	96.8	-	
10	L. plantarum	77.4	87.6	92.5	92.5	94.2	92.2	94.0	96.1	97.7	-
11	Str. bovis	77.2	88.3	90.3	90.5	92.0	89.5	91.8	92.0	92.6	93.2

Table 4.2

Percentage similarity between species of the genera *Lactococcus* and *Enterococcus*. (Mean pairwise distances between taxa, with adjustment for missing data, calculated using PAUP v.3.0.)

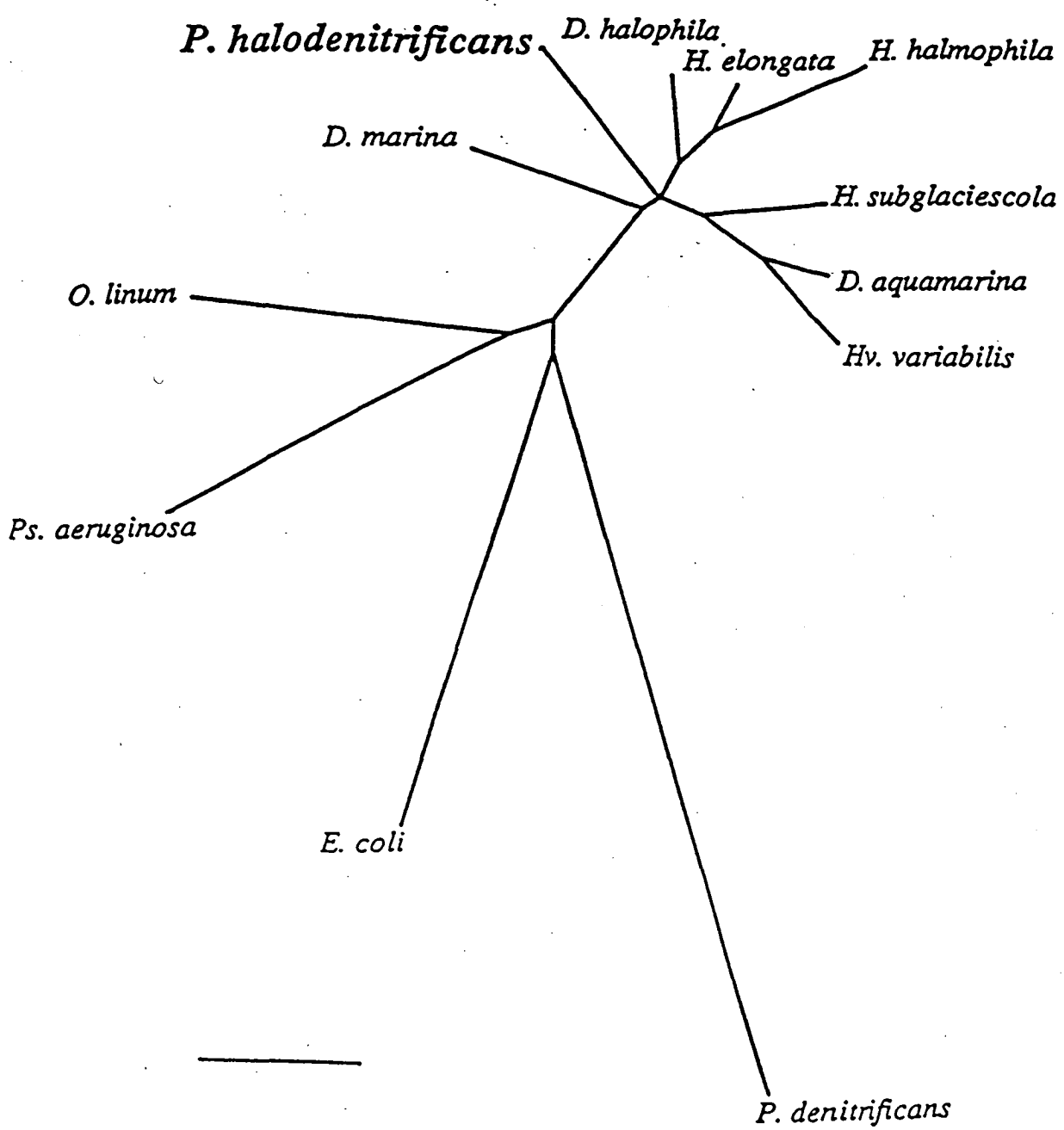

Fig. 4.1
Phylogenetic tree derived from 16S rRNA sequences subjected to distance analysis, showing the relationships between *P. denitrificans*, *P. halodenitrificans*, members of the family Halomonadaceae and other species of the gamma subclass of the Proteobacteria. Bar = 0.03 evolutionary distance units as calculated by the Jukes-Cantor method.

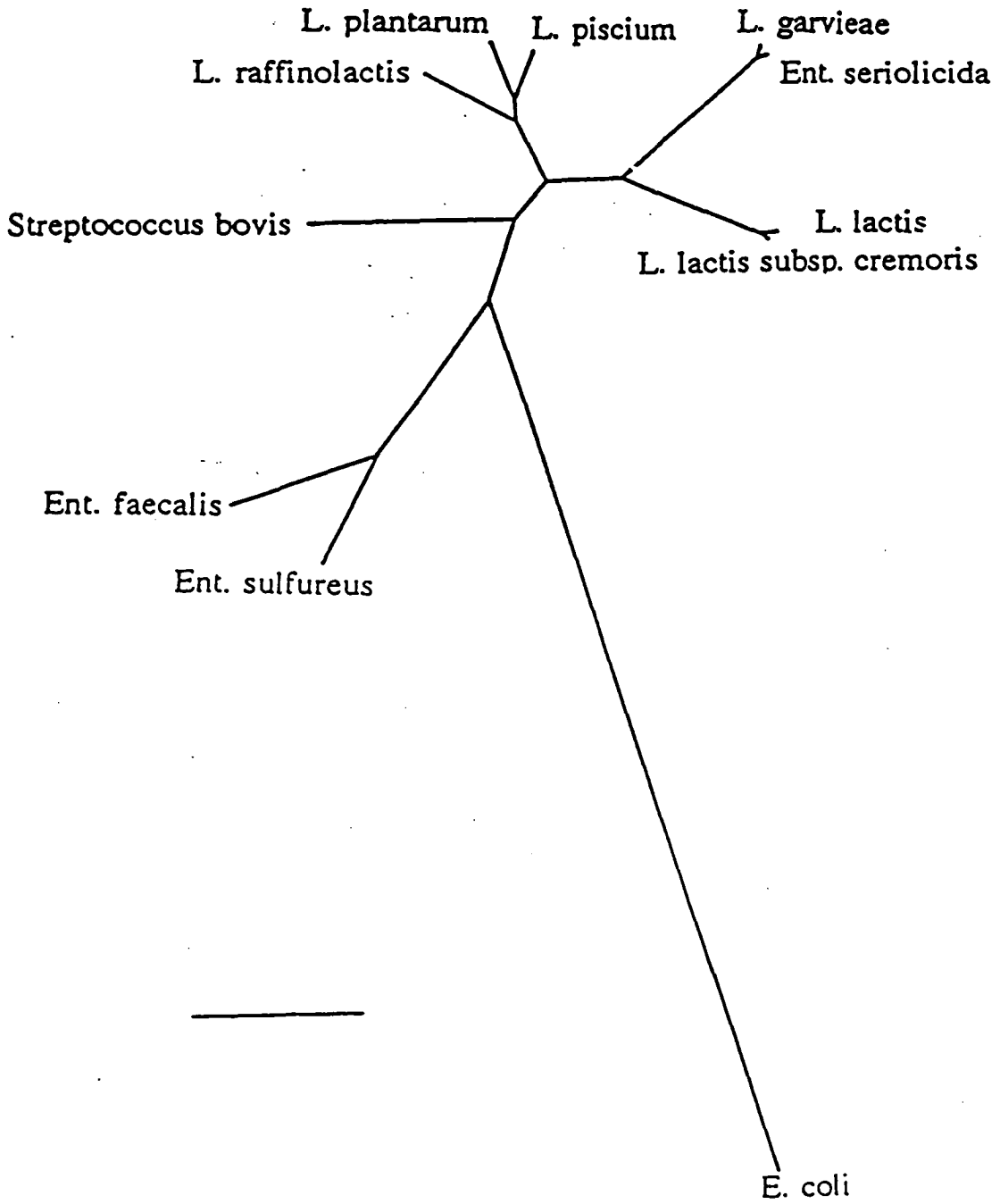

Fig. 4.2
Phylogenetic tree derived from 16S rRNA sequences subjected to distance analysis, showing the relationships between members of the genera *Lactococcus* and *Enterococcus*. Bar = 0.03 evolutionary distance units as calculated by the Jukes-Cantor method.

5. DISCUSSION

5.1 *Paracoccus halodenitrificans*

Comparisons of 16S rRNA sequence and signatures of *P. halodenitrificans* (ATCC13511^T) with those of members of the family Halomonadaceae indicate that *P. halodenitrificans* is most appropriately assigned to this family. It has therefore been proposed (Miller et al., 1993) that *P. halodenitrificans* be transferred to the family Halomonadaceae in the γ-subclass of the Proteobacteria.

Of the comparisons made, *P. halodenitrificans* is most closely related to *Deleya halophila* with a sequence similarity of 94.3%. Although the correlation of DNA-DNA reassociation and 16S rRNA sequence similarity is not linear, it has been suggested on the basis of compiled data that chromosomal reassociation will be >60% for bacteria that differ by less than 3% in 16S rRNA sequence (Stackebrandt & Goebel, 1994). By this criterion, *P. halodenitrificans* is a distinct species within the Halomonadaceae. Within the family, 16S rRNA sequence similarities range from 97.6% and 92.5% (disregarding comparisons of probable synonyms *D. aquamarina* and *H. meridiana*).

It is not possible at this time to specify allocation of *P. halodenitrificans* to one of the three genera of the family Halomonadaceae. The genera *Deleya*, *Halovibrio* and *Halomonas* have been shown to be indistinguishable by phenotypic, chemotaxonomic and molecular criteria (Dobson, 1988; Franzmann & Tindall, 1990; James et al., 1990; Skerratt et al., 1991; Dobson et al., 1993). Until the internal structure of the family Halomonadaceae is clarified at the genus level, assigning *P. halodenitrificans* to a genus would be an arbitrary and gratuitous exercise.

5.2 *Aeromonas salmonicida*

5.2.1 Taxonomic position of *A. salmonicida* strain 93/1061

The well established definition of *A. salmonicida* (Popoff, 1984) cites two characteristics by which the recognised atypical subspecies are

distinguishable. Subspecies *achromogenes*: is negative for aesculin hydrolysis and mannitol fermentation. Subspecies *masoucida* is positive for aesculin hydrolysis and mannitol fermentation. The production of indole is positive for *masoucida* and variable for *achromogenes* Austin et al. (1989) have disputed these findings, assessing *masoucida* as aesculin negative, and *achromogenes* as mannitol positive. The two subspecies are thereby identical by these criteria. Indole production is given as the definitive character (*masoucida* positive, *achromogenes* negative).

Numerical taxonomic comparisons of the subspecies of *A. salmonicida* (Austin et al., 1989) have clustered subspecies *salmonicida* with *masoucida*, and subspecies *smithia* with *achromogenes*. The authors strongly advocate the continued separation of the two subspecies *achromogenes* and *masoucida* on this basis.

DNA-DNA homology data do not effectively separate the two subspecies *achromogenes* and *masoucida* (MacInnes et al., 1979; Belland & Trust, 1988), though data are conflicting (Austin et al., 1989). It is interesting to note discrepancies in the genomic homology between *A. salmonicida* subspp. *salmonicida* and *achromogenes*. Belland & Trust (1988), consistent with earlier findings (MacInnes et al., 1979), give reassociation values for these subspecies at 70%, and between subspecies *salmonicida* and *masoucida* of 100%. They suggest that the subspecies *achromogenes* and *masoucida* be combined on the basis of DNA-DNA reassociation values of each with *A. salmonicida* subsp. *salmonicida* that imply genetic identity. Austin et al. (1989) cite a level of DNA reassociation of 44% between *A. salmonicida* subspp. *salmonicida* and *achromogenes*, reflecting a genetic relatedness well below conspecific, and reassociation value of 76% for subspecies *salmonicida* and *masoucida*. A possible source for these discrepancies is the different hybridisation protocols used. There is also variation between groups in the organisms studied. The strain of subspecies *salmonicida* used as probe differs in each study and the strain of subspecies *masoucida* used by Belland & Trust differs from that used by the other two groups. Even the terms used to describe the data presented are inconsistent. Both Belland & Trust (1988) and MacInnes et al. (1979) describe their results as "DNA homology" values expressed as relative binding ratios. However, relative binding ratios are not a measure of DNA homology but of genomic reassociation. Austin et al. (1989) refer to their data as reassociation values. These inconsistencies highlight a major

problem with DNA-DNA hybridisation analysis: reproducibility of results between laboratories and with different techniques.

Genomic identity of the subspecies *achromogenes* and *masoucida* is an indirect inference based on the similar hybridisation values of each with *A. salmonicida* subsp. *salmonicida* (70% and 90-100%, respectively), with *A. hydrophila* and with *A. salmonicida* subsp. "*nova*" (MacInnes et al., 1979; Belland & Trust, 1988; Austin et al., 1989). Both subspecies are considered conspecific with all strains of *A. salmonicida.* They are each genetically equally distant from other atypical *A. salmonicida* strains and from *A. hydrophila* (Belland & Trust, 1988). However, because two strains are each considered conspecific with a third on the basis of DNA-DNA reassociation, their mutual conspecificity can only be justified if the reassociation values are very high. The inference of conspecificity here is, therefore, precarious and especially so when data are contentious (Austin et al., 1989). It is unfortunate that there is no direct hybridisation data between genomes of the atypical subspecies of *A. salmonicida* which might clarify their relationship. This illustrates the other major drawback in DNA-DNA reassociation analysis: binary measures of similarity allow direct comparison of many strains only with a vast and sometimes prohibitive amount of experimentation.

Subspecies *masoucida* and *achromogenes* have similar DNA G+C base composition (60.9 mol% and 59.7 mol%, respectively). They more closely resemble *A. hydrophila* (60.6 mol%) in this respect than subspecies *salmonicida* or "*nova*" (approx. 55 mol%)(Austin et al., 1989). *A. salmonicida* strain 93/1061 has a G+C content of 57-59 mol% (J. Carson, pers. comm.).

Sequences of the 16S rRNA genes of subspecies *masoucida* and *achromogenes* are identical. *A. salmonicida* strain 93/1061 shares 100% 16S rRNA sequence identity with both. There are two 16S rRNA sequence differences between *A. salmonicida* subspp. *salmonicida* and *masoucida / achromogenes /* strain 93/1061 = 99.9% 16S sequence relatedness (Martinez-Murcia et al., 1992a). DNA-DNA reassociation of the three recognised subspecies is high enough to classify them as conspecific (within the limitations described above). The DNA-DNA integrity of the subspecies *salmonicida* (and including *masoucida,* according to MacInnes

et al. [1979]) is approx. 100%. Therefore, the subspecies are by currently accepted criteria genetically indistinguishable.

Lack of 16S rDNA sequence or chromosomal hybridisation data on subspecies *smithia* precludes its genetic comparison with strain 93/1061. Phenotypically, however, the former does not produce indole, degrade aesculin or, uniquely for atypical subspecies in this analysis, ferment mannitol (Austin et al., 1989).

In the light of these data, the taxonomic position of strain 93/1061 seems to depend solely on assessment by comprehensive numerical taxonomy. The two subspecies with which it shares 16S rRNA sequence identity are defined and distinguished in this way. The genetic homogeneity of the species *A. salmonicida* strongly suggests that the third atypical subspecies *smithia* will share this genetic identity. The taxonomic position of strain 93/1061, therefore, will be established in relation to these subspecies by the criterion of numerical taxonomy.

5.2.2 Appropriate application of 16S rRNA sequence comparisons to taxonomy

The definition of a bacterial species is currently based on DNA reassociation data which measures the sequence similarity between whole genomes of bacterial strains (Wayne et al. 1987). However, most recent taxonomic studies have adopted 16S rRNA gene sequences as the basis of comparative phylogeny. These sequence comparisons allow a broad phylogenetic assessment of relationships with a single analysis. Until recently, apart from some exceptional cases of brachytelic evolution, there has been little indication that the evolution of the rRNA genes does not reflect the evolution of the genome as a whole. Indeed, this has been one of the basic assumptions of 16S rRNA-based phylogenetic inference.

Comparative 16S rRNA gene sequences have confirmed the fundamental homogeneity of the genus *Aeromonas* and the species *A. salmonicida* (Martinez-Murcia et al., 1992a). The 16S rRNA sequences of 20 *Aeromonas* strains, including 10 type strains, show between 97.8-100% homology (= 0-32 base differences over 1502 nucleotides). However, the internal structure of the genus *Aeromonas* cannot be defined by 16S rRNA

sequence comparisons. The nucleotide differences in the gene are statistically insignificant, though they may be experimentally and evolutionarily meaningful (Fox et al., 1992). They offer no means of drawing any consistent taxonomic conclusions. The susceptibility to distortion of such a finely discriminated definition as the nucleotide differences in the 16S rRNA gene mitigates its usefulness in the aeromonads.

Because the intrageneric levels of 16S rRNA sequence difference in the genus *Aeromonas* are statistically trivial, any meaningful comparison with DNA-DNA homology values for this genus are specious. This is exemplified by the lack of congruence between the two data sets in the work of Martinez-Murcia et al. (1992a). rRNA sequence similarities are often in contradiction with hybridisation data and phenotypic characterisations for the same species or subspecies. For instance, the species *A. hydrophila* subsp. *hydrophila* and *A. media* have only three base differences in their 16S rRNA genes. They are, however, assigned to different DNA-DNA hybridisation groups, consistent with the phenotypic description of them as individuated species. The species *A. caviae* and *A. trota* have only a single base difference in their 16S rRNA gene, which in no way reflects the 30% reassociation of their genomic DNA under optimal conditions (Carnahan et al., 1991). On the other hand, two strains of *A. hydrophila* subsp. *anaerogenes* differ by 0 and 2 bases from the type strain of *A. caviae* in keeping with high levels of DNA homology that suggest these strains are effectively conspecific (Hickman-Brenner et al., 1988). In this manner, many of the species of *Aeromonas* are individuated by remarkably few differences in the 16S rRNA gene, despite apparent genomic differences sufficient to justify species status (ie <70% DNA reassociation [Wayne et al., 1987]). It has been suggested that *A. trota* and *A. enteropelogenes* are "identical" (*sic*) on the basis of identical 16S rRNA sequences; and that *A. ichthiosmia* and *A. veronii* are "identical" on the same basis (Collins et al., 1993). DNA-DNA hybridisation data for the former pair is lacking and for the latter pair suggests non-identity. Given the indeterminacy of 16S rRNA sequence comparisons in this genus, this proposition seems premature.

DNA-DNA hybridisation will not detect similarity between organisms that differ in overall genomic sequence by more than 15%. However, DNA-DNA hybridisation is the definitive basis for resolving phylogeny at the

intrageneric level where genome differences are typically well within these limits. Therefore, within the genus *Aeromonas*, DNA-DNA hybridisation data should confidently represent the internal relations of the genus. 16S rRNA sequence divergence does not reflect overall genomic similarity amongst very closely related strains, however, tempering one of the fundamental premises upon which is based the use of 16S rDNA comparisons as a phylogenetic measure.

Similar inconsistencies have been reported for the genus *Bacillus* (Fox et al., 1992), the genus *Mycobacterium* (Baess, 1983; Boddinghaus et al., 1990) and the genus *Serpula* (Stanton et al., 1991). 16S rDNA sequence near-identity (98.5-99.8%) is not consistently reflected in DNA-DNA reassociation values that define conspecific strains.

It has long been recognised that genomic and 16S rRNA sequence comparisons resolve phylogeny with maximal sensitivity at different taxonomic levels. Where there is overlap in their application, however, the relationship of these parameters has not been clear. Inconsistencies between these data sets for the genera *Bacillus*, *Mycobacterium* and *Serpula* prompted Fox et al. (1992) to propose that "when a small number of [16S rRNA] sequence differences are found ..... it is not at all certain that DNA-DNA hybridisation data make a meaningful distinction [between species]". On the contrary, in these circumstances it is the reassociation values that make the more meaningful distinction of species. In recently evolving groups, rRNA sequences cannot be used to define intrageneric relationships (Stackebrandt, 1992). Overall genomic evolution will accrue more sequence differences over a short period, under the intense selective pressure that gives rise to the characteristic phenotypic diversity of these taxa, than will a highly conserved gene responding to random genetic drift. Correspondingly, in older lineages, directed evolution will have reached a plateau and stabilised with adaptation in the earlier history of the taxon, while the drift of its conserved genes has continued in its "clocklike" regular way. Consequently, there is greater correspondence between the two "chronometers" in these older taxa. There is no *a priori* reason to assume that the rate of genomic evolution should be constant between or within taxa, or over time.

The issue seems to be whether genetic relatedness is to be a measure of time or of genetic distance: the former measured by the sequences of the

16S rRNA molecule, and the latter by the overall sequence differences of whole genomes (Fox et al., 1980). Currently, the officially recognised parameter is DNA-DNA reassociation (Wayne et al., 1987). As described above, this fails at genetic distances beyond those separating genera. At these distances, however, the relationships derived from both techniques converge, though they may not meaningfully meet. Ribosomal RNA sequence comparisons often reflect the relationships described by chromosomal reassociation values. That is, at certain phylogenetic depths, rRNA sequence comparisons become measures of both genetic distance and time. However, as there is an upper limit to the usefulness of DNA-DNA homology analysis, there is a lower limit to the usefulness of 16S rRNA sequence analysis. For very recently speciated groups of bacteria, the random drift of sequence in ribosomal RNA genes provides insufficient distinction for taxonomic purposes.

Genetic data for the subspecies of *A. salmonicida* are less internally inconsistent than for the rest of the genus. *A. salmonicida* subsp. *achromogenes* and *A. salmonicida* subsp. *masoucida* have identical sequences and implied chromosomal reassociation values high enough to have prompted several authors to suggest that their distinction is spurious (MacInnes et al, 1979; Belland & Trust, 1988). *A. salmonicida* subsp. *salmonicida* 16S rRNA sequence differs from the other subspecies in only 2 bases, with corresponding reassociation values of 70% and 90-100% (*achromogenes* and *masoucida* respectively) at an optimal reassociation temperature of 70°C (MacInnes et al., 1979: Belland & Trust, 1988). Both data sets confirm conspecific status. However, because the phylogenetic depth of the genus is obviously so shallow, 16S rRNA sequence comparisons are not a true measure of relatedness for these strains. Furthermore, measures of DNA-DNA reassociation are indirect, as described above, and also contradictory (Austin et al., 1989), leaving the inclusion in the species of the subspecies *achromogenes*, at least, open to dissent.

5.3.2 What does this mean for *A. salmonicida* strain 93/1061?

When strain 93/1061 was first isolated, it was tentatively but confidently identified as belonging to the non-motile aeromonads, circumscribed by the species *A. salmonicida*. It has characteristics common to atypical members of the species, with which it shares 16S rRNA sequence identity.

These data are consistent with the fact that there has been no evidence for any presence of *A. salmonicida* subsp. *salmonicida* in Australia, and that this subspecies is considered to be exotic (Carson, 1994).

However, given the dubiousness of defining a species within this group on the basis of 16S rRNA sequence, to assign strain 93/1061 to the species or to a particular sub-species without DNA-DNA homology data to corroborate the placement is premature. A noteworthy 16S rRNA sequence identity exists between *A. salmonicida* subspp. *achromogenes* and *masoucida* and *Aeromonas* sp. CIP 7430. The latter strain has previously been allocated to a different hybridisation group and is thereby more closely related to *A. hydrophila* than to *A. salmonicida* (Kuijper et al., 1989; Martinez-Murcia et al., 1992a). This would seem to further impair 16S rRNA sequence identity as a valid criterion for species identity at the phylogenetic depth of this genus.

In summary, the final assignment *A. salmonicida* strain 93/1061 must await:
1. DNA-DNA hybridisation analysis with other subspecies of the genus, to define conclusively its conspecificity with them
2. comprehensive numerical taxonomy by which it may be distinguished from other subspecies of *A. salmonicida.*
Only then will it be possible to establish whether this bacterium is an endemic subspecies of *A. salmonicida.*

5.2.4 Probes

The strong uniformity of sequence in the 16S rRNA gene within the genus *Aeromonas* means that while its use as the target for species-specific oligonucleotide probes is feasible (Barry et al., 1990; Dorsch et al., 1994), that for a subspecies-specific probe is not. Primer As1 does not distinguish subspecies of *A. salmonicida*, nor distinguish them from *A. sobria* or *Aeromonas* sp. ATCC 49568. The one base mismatch with *A. eucrenophila*, *A. jandaei*, *A. veronii* and *A. schubertii* is insufficient to reliably discriminate the species even under stringent conditions, (though some sanguine workers would suggest otherwise [Wallace et al., 1979]). Primer As2 is definitive for *A. salmonicida*, but will not distinguish its subspecies. Because these subspecies are pathogenic to salmonid fish to various

degrees, a probe is required that can identify the organisms to subspecies level. 16S rRNA-directed probes are ineffective to this purpose.

Alternative molecular identification systems have been proposed. A random genomic DNA fragment from *A. salmonicida* has been used as hybridisation probe to detect the species (Hiney et al., 1992), and PCR primers designed from the terminal sequences of this probe used as diagnostic PCR primers (Hiney et al., 1992; Mooney et al., 1995; O'Brien et al., 1994). It is possible that this approach is applicable to the distinction of subspecies.

Aeromonas species are distinguishable when restricted genomes are probed with fragments of the *E. coli* 16S rRNA gene (Martinetti Lucchini & Altwegg, 1992), though *A. salmonicida* was not included in this analysis. Genomic DNA from strains of *A. salmonicida* was restricted with seven enzymes and probed with four plasmid-borne sequences (Hennigan et al., 1988), but the genetic integrity of the species is such that no sequence variation was revealed. *A. salmonicida* strain 93/1061 was distinguishable from other atypical strains, however, by a unique restriction endonuclease fingerprint (Whittington et al., in press). Sensitivity of analysis by random amplification of polymorphic DNA (RAPDs) is comparable to RFLP analysis (Palittapongarnpim et al., 1993). Therefore, genomic sequence divergence of these subspecies should also be amenable to distinction by RAPDs. Subspecies-specific bands detected on electrophoretic separation of amplification products can be purified and cloned into a vector to act as a diagnostic probe for a particular subspecies (Fani et al., 1993).

Sequences within the surface array protein gene, the virulence factor associated with *A. salmonicida*, were used as primers for a diagnostic PCR (Gustafson et al., 1992). This elegant assay not only specifies *A. salmonicida* but can predict the potential pathogenicity of the strain by assessing the status of the virulence determinant. Once again, however, subspecies are not identified.

The sequences of non-coding DNA diverge more rapidly than gene sequences and are therefore more likely to distinguish genetically close groups. Therefore, oligonucleotide probes targeting the ribosomal intergenic spacer region between the 16S and 23S rRNA genes are likely

to discriminate subspecies and strains of *A. salmonicida* (Powell et al., 1993). Though there are no functional constraints maintaining comparable sequence in non-coding regions of the several rRNA cistrons of an organism, sequence similarity is sustained (Barry et al., 1991), presumably through mechanisms of concerted evolution, as in most tandemly repeated gene families. Sequence heterogeneity, therefore, does not interfere with easy access to DNA sequences in these regions.

5.3 *Enterococcus seriolicida*

The contention that *Ent. seriolicida* and *L. garvieae* are very closely related is supported by analysis of 16S rRNA sequences of members of the genera *Lactococcus* and *Enterococcus*. The phylogenetic tree of Fig. 4.2 places *Ent. seriolicida* on a branch with *L. garvieae* indisputably among the lactococci. Whether there is sufficient genetic or phenotypic justification for attributing to *Ent. seriolicida* full species or subspecies status will be discussed.

5.3.1 16S rRNA sequences of *Ent. seriolicida* and *L. garvieae* compared

5.3.1.1 sequence differences

There are seven apparent differences between the 16 rRNA sequences of *Ent. seriolicida* and *L. garvieae*. Whether or not this is a numerically significant degree of differentiation, it is useful to assess these differences individually.

When seeming aberrations in rRNA sequence data occur, it is customary to test their veracity by examining their effects on the pairing of bases across helices of the rRNA molecule. Watson-Crick base-pairing in the double-stranded regions of the rRNA molecule is not without exception. However, base changes across helices are often cooperative, with a base substitution at one position complemented by a simultaneous base-change at a second position so that canonical pairing across the helix is maintained. This is important for the conformational stability of the rRNA molecule imposed by its secondary structure. Single base changes that transgress the base-pairing demanded by rRNA secondary structure are therefore noteworthy. The *L. garvieae* sequence, alone of the gram

positive cocci, transgresses Watson-Crick pairing at position 193b (difference 2) in helix 10. Furthermore, the single-base deletion cited for *L. garvieae* at position 1027 (difference 4) challenges the integrity of helix P37-2 (Neefs et al., 1993). Keeping in mind that the secondary structure models of rRNA molecules are largely conjectural and subject to constant reassessment, these are nonetheless significant aberrations.

Other contentious sequences lie in single-stranded parts of the molecule. While these regions of the molecule are not constrained by considerations of secondary structure, it is in these loops that much of the invariant sequence occurs. Interactions between the 16S rRNA molecule and the other components of the translational apparatus take place largely at these sites (Nomura et al., 1969) and the RNA sequence is conserved accordingly.

Of the disparities between the sequences of *L. garvieae* and *Ent. seriolicida* itemised above (4.3.1), it would not be unreasonable to suggest that differences 5, 6 and 7 constitute possible mistakes in the *L. garvieae* sequence. In each case does the *L. garvieae* sequence contradict the sequence conserved across not only the genus *Lactococcus,* but also through most of the prokaryote kingdom (Neefs et al., 1993). Difference 2 also suggests a possible mistake in the *L. garvieae* sequence. The deletion of 4 is anomalous, as described above, and also unusual in that it has arisen twice, once in *L. raffinolactis* and again in *L. garvieae.* Furthermore, this deletion occurs in the 16S rRNA sequence of *Lactococcus lactis* subsp. *lactis* (NCDO 2118) published in the literature (Collins et al., 1989) but not in the sequences of all strains reported in the genomic databases (eg. strain 7962, Salama et al., 1991). These inconsistencies suggests that the deletion is a sequencing artefact misconstrued from the compression of tandem cytosines. The 16S rRNA gene of *L. garvieae* (and those of *L. raffinolactis* and *L. lactis*) were sequenced by reverse transcription (Collins et al., 1989). This method is known to result in a proportion of errors due to the tendency of reverse transcriptase to founder at regions of secondary structure

There is inevitably a degree of uncertainty associated with any rRNA sequence presented (Fox et al., 1992). As well as experimental artefacts, there is the possibility of microheterogeneity of sequences when multiple ribosomal RNA cistrons are present. Therefore, when sequence

differences are few, their interpretation must be undertaken with extreme circumspection.

Of the seven noted sequence disparities, five are open to doubt. (The other two probably come down to a matter of opinion). Countering these arguments is the fact that in sequencing the 16S rRNA molecule of *L. garvieae*, Collins et al. (1989) examined the primary structure of molecules from two strains, NCDO 2155^T and NCDO 2156. These two strains differed by only one nucleotide. (The nucleotide was, unfortunately, not specified). This could be construed as an effective verification of the sequence published. All polymerases, however, and reverse transcriptase in particular, are susceptible to replication errors and certain errors can be recurrent (DeBorde et al., 1986). Sites of robust secondary structure can produce artefacts in the sequence, as can regions of multiply repeated tandem bases (especially cytosine). It is not unusual for a particular position in a sequence to be refractory to clarification. Many an alignment has a column of homologous "n"s through several related species.

5.3.1.2 sequence similarities

Of the sequence similarities noted (Appendix 2), the sequences that are unique to the two strains (similarity 1), the singular 5 pair extension to helix 6 that they share (similarity 2) and the higher order structural changes to the primary sequence (similarity 4i) common to both are significant signatures for *L. garvieae* and *Ent. seriolicida* within the lactococci.

5.3.2 Taxonomic implications

5.3.2.1. genetic status of *Ent. seriolicida*

If the seven 16S rRNA sequence differences between *Ent. seriolicida* and *L. garvieae* are real, they are still too few to be statistically meaningful (Fox et al., 1992). As illustrated for the genus *Aeromonas*, the age or phylogenetic depth of a genus, (the period over which its members have speciated) is reflected in the degree of difference in its 16S rRNA molecular sequences. The most closely related species in the genus *Lactococcus*, *L. plantarum* and *L. piscium*, differ by 41 nucleotides over the

effectively complete 16S rRNA gene sequence (Williams et al., 1990). Therefore, in the context of this genus, species are separated by at least 96.7% 16S rRNA sequence similarity (= 41 base differences). The seven base differences between *Ent. seriolicida* and *L. garvieae* constitute a 99.5% 16S rRNA gene sequence similarity. It is unlikely that full species status is warranted.

Within the genus *Lactococcus*, the variation in 16S rRNA sequence between synonymous species and between strains accorded subspecies status is comparable and statistically insignificant. *L. lactis* subspp. *lactis* (NCDO604^T) and *hordniae* (NCDO 2182) are separated by only 2 nucleotides (Collins et al., 1989). *L. lactis* subsp. *lactis*(NCDO604^T) and subsp. *cremoris* (NCDO607^T) are separated by 9 nucleotide differences in the sequences presented in the RDB. The 16S rRNA sequence of a strain of *L. garvieae* (NCDO 2156) differed from that of the type strain (NCDO 2155^T) by a single nucleotide. Given these precedents, the seven or fewer differences in 16S rRNA sequence of *L. garvieae* and *Ent. seriolicida* could justify either synonymy or subspecies status. Sub-division of the species, however, requires corroborative DNA-DNA hybridisation analyses within the context of the whole genus and clear phenotypic distinctions between the two organisms. As in the case of the subspecies of *L. lactis* (Schleifer et al., 1985), subspecies status should be accorded only with strong supporting phenotypic and biochemical differentiation.

5.3.2.2 phenotypic status of *Ent. seriolicida*

The phenotypic characters that have been used to identify strains of these species have included capricious ones (Table 5.1).

	L. garvieae		*Ent. seriolicida*				
	1.^{T}	2.^{T}	3.^{T}	4.^{T}	5.^{T}	6.	7.
Growth at 45°C	-	-	+	+	-	-	-
α-haemolysis	-	+	+	+	+	+	+
Growth at 6.5% NaCl	ND	+	+	+	+	late +	late +
Group N antigen	+	ND	-	ND	?	-	ND
Growth at pH 9.6	ND	weak, delayed	+	+	+	ND	weak, delayed

Table 5.1 - Phenotypic characters of *L. garvieae* and *Ent. seriolicida*

1. original reference
2. Toranzo et al., 1994
3. Kusuda et al., 1991
4. Toranzo et al., 1994
5. Schmidtke & Carson, 1994
6. endemic strain, Carson et al., 1993
7. strain from Spanish turbot, Toranzo et al., 1994
8. strain from Spanish trout, Domenech et al., 1993

ND - no data available

T - profile of the type strain

Of these data, *L. garvieae* and *Ent. seriolicida* are distinguished by:

1) *growth at 45°C*

These data appear recalcitrant. *L. garvieae* would seem clearly negative; *Ent. seriolicida* positive. Experimental discrepancies seem unlikely when results derive from the same laboratory (Toranzo et al., 1994). Only the results provided by DPIF, Tas. (Schmidtke & Carson, 1994) suggest that *Ent. seriolicida*, like *L. garvieae*, does not grow at 45°C.

An estimated 95% of clinical isolates of *L. garvieae* were shown to grow at 45°C after extended incubation (>48 hr). However, growth at 45°C was sufficiently arrested over shorter periods that it was used as the diagnostic distinction between *L. garvieae* and enterococci (Elliott et al., 1991). It is tempting to presume, on this basis, that experimental variation is responsible for inconsistencies among *L. garvieae* strains. The classic protocol for generic identification of the Gram positive cocci (Facklam & Washington, 1991) explicitly states that growth at 45°C should be observed for up to seven days. Facklam et al. (1989), presumably using the classic protocol, found 25% of lactococci examined grew at 45°C, undermining the traditional distinction of the genus *Lactococcus*. However, use of the term "extended incubation" to describe >48 hours (Elliott et al., 1991) suggests that incubations are typically considered acceptable for shorter periods. Very little of the literature specifies the period over which this test, in particular, is undertaken. The temperature tolerance tests by which strains of *L. garvieae* have been defined and confused are also affected by base medium, type of incubation (water bath or air incubator) and inoculum (Facklam et al., 1989). Until all experimental parameters are standardised, it is difficult to reliably compare results derived from insufficiently described work.

Both empirical (Ikawa et al., 1986; Jensen et al., 1987; Graham & Lund, 1993) and model-based (Ratkowsky et al., 1991; Ratkowsky et al., 1995) studies have indicated that, at the limits of growth, variance in generation times increases. Consequently, growth can be predicted with decreasing reliability as the stringency of the bacterial environment increases. Growth at 45°C, therefore, is an unfortunate choice as the definitive character for these genera.

From the data cited, two conclusions can be drawn. It is possible that only some strains of *L. garvieae* grow at 45°C. Alternatively, *L. garvieae* grows at 45°C, but some strains do so more slowly than others. The law of the excluded middle need not apply: both conclusions may be legitimate. Growth at 45°C has been a criterion for genus identification for a long time. However, it is neither a robust nor a reliable arbiter of taxonomic status here. Whether standardised protocols provide reproducible descriptions of strains remains to be seen. In the meantime, determination of a more conclusive character by which to differentiate the enterococci and the lactococci seems mandatory.

2) *group N antigen*

L. garvieae is conventionally described as positive for Lancefield group N antigen, in keeping with the other lactococci. However, in transferring the species from the genus *Streptococcus* to the genus *Lactococcus*, Schleifer et al. (1985) describe the new species *L. garvieae* as group N positive for some strains only (including NCDO 2155^{T}). Serological differences between *Ent. seriolicida* and *L. garvieae* could, therefore, signify merely a difference in strain.

3) *α-haemolysis*

Haemolysis has not been cited as characteristic for any of the lactococci, except some weak α-haemolysis in strains of *L. lactis*. However, the consensus in these data suggests that re-evaluation of the type strain of *L. garvieae* may be warranted. Alternatively, *L. garvieae*, like *L. lactis*, may be polymorphic for this trait. The suggestion of β-haemolysis (Domenech et al., 1993) is inconsistent with both the other data cited here and its widely documented absence among the lactococci.

4) *chemotaxonomy*

L. garvieae has a unique and therefore definitive constituent of its cell wall, the peptide lys-ala-gly-ala. Its occurrence in *Ent. seriolicida* would be an extremely convincing chemotaxonomic corroboration of conspecificity.

5.3.3 Sub-species status

The characteristics that differentiate the *L. garvieae* and *Ent. seriolicida* are significant (growth at 45°C, for instance) but inconclusive. Phenotypes are often contradictory and ambiguous, and reproducibility of characters is not reliable. These inconsistencies may be experimental artefacts, arising from variations in the methods of data collection. In this case, 16S rRNA-based genetic identity would signify species synonymy of *Ent. seriolicida* with *L. garvieae*.

If, however, these variations are real, lack of clear phenotypic definition suggests two possibilities. The various strains of *L. garvieae* may be polymorphic for these traits. Standardisation of test procedures and strain descriptions would allow systematic assessment of such polymorphisms. Alternatively, the cline in these characters is so finely discriminated between strains that tests are insufficiently rigorous to detect them with consistent reproducibility. Either explanation would suggest that sub-dividing the species is unjustified.

5.4 SUMMARY

16S rRNA sequence comparisons allow an assessment of whether taxa are phylogenetically homogeneous (Stackebrandt & Goebel, 1994). In this way misclassifications are identifiable, as in the cases of *P. halodenitrificans* and *Ent. seriolicida*.

* *Paracoccus halodenitrificans*
Sequence analysis has allowed a more coherent taxonomic placement of the species, involving reallocation to both family and subclass. The position of *P. halodenitrificans* in relation to other members of the family

is clear. 16S rRNA sequence similarity between *P. halodenitrificans* and its nearest relative, *D. halophila*, is 94.3% which by several computations (Amann et al., 1992; Fox et al., 1992; Stackebrandt & Goebel, 1994) confers species status. The Halomonadaceae is presently divided into three genera, though by all classificatory criteria only a single genus is justifiable (Dobson et al., 1993). Consequently, *P. halodenitrificans* can be characterised by molecular criteria to genus level, albeit awaiting reorganisation of the internal structure of the family.

* *Aeromonas* sp.
The relationship between genomic sequence comparisons and comparisons of rRNA gene sequences is not intuitively obvious. However, as the database of gene sequences has grown, it has become clear that these parameters do not correlate in a linear way. Furthermore, 16S rRNA sequence similarities cannot be standardised across taxa to give an unambiguous definition of species.

* *Enterococcus seriolicida*
As with the re-classification of *P. halodenitrificans*, 16S rRNA sequence comparisons have allowed clarification of the taxonomic position of *Ent. seriolicida* and revealed its probable synonymy with *L. garvieae*.. Whether subspecies status is warranted depends on clarification of phenotypic criteria. The Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics (Wayne et al., 1987) recommends "subspecies" designations for genetically close organisms with divergent phenotypes. Furthermore, the Committee suggests that "there is some evidence, based on frequency distribution of ΔT_m values in DNA hybridisation, that the subspecies concept is phylogenetically valid", citing the need for further guidelines in the designation of subspecies.

In 1980, Fox et al. proposed that the degree of 16S rRNA sequence similarity between species is a function of the "phylogenetic depth" of the taxon to which they belong. Ancient genera are constituted of species with high levels of sequence dissimilarity. Ribosomal RNA sequence of recently evolved genera have far fewer differences. Although 16S rRNA sequence comparisons cannot be given a direct role in bacterial species definition, it has been proposed that their part in polyphasic taxonomy is as a measure of "phylogenetic depth", of the relative age of the taxon under consideration. Sequence similarity measures of >97.5% would

suggest that the strains under consideration are sufficiently closely related that sequence data may fail in intrageneric distinctions (Stackebrandt & Goebels, 1994). DNA-DNA reassociation analysis is necessary under these circumstances to accurately assess inter-species relationship. From the example of the genus *Aeromonas* it is clear that the phylogenetic depth of a genus imposes constraints upon the taxonomic parameters that can be usefully employed to examine intrageneric structure.

The 16S rRNA gene of the genus *Aeromonas* has recently been subject to a different sort of scrutiny with interesting and perhaps far-reaching results. Comparison of the sites of sequence differences in the gene has suggested that the gene itself is the hybrid product of one or several crossing-over events between different genes (Sneath, 1994). This implies horizontal gene transfer between species, whether by plasmid vector or other mechanism/s. The sequence similarities between species will therefore be distorted, and almost inevitably under-estimated. If this hypothesis is correct, there may be consequences also for the concept of phylogenetic depth. Phylogenetic depth is presently inferred from the level of 16S rRNA sequence similarity within a genus. If high levels of similarity are actually the result of horizontal gene transfer, with genetic crossing-over and gene conversion, followed by fixation of the hybrid gene, there is no obvious necessary correlation with the age of the genus in which this occurs. The phenotypic diversity said to typify "shallow" genera may have its basis in a function common to the genetic flexibility that allows these events. The correlation with generic age may be indirect or gratuitous. It may be that only recently speciated groups maintain enough genetic semblance to allow this sort of genetic interchange. Sneath (1994) further suggests that this phenomenon reasonably explains the very short or long branches in some phylogenetic trees, for which different rates of evolution have been invoked for different organisms.

Should the ribosomal RNA genes be shown available to these sorts of genetic permutations, the premise that their phylogenies truly represent the phylogenies of the bacteria containing them is compromised. The use of 16S rRNA sequence comparisons in taxonomy should then be verified even more stringently with a polyphasic suite of characters.

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Woese, C. R., W. G. Weisburg, C. M. Hahn, B. J. Paster, L. B. Zablen, B. J. Lewis, T. J. Macke, W. Ludwig & E. Stackebrandt. 1985a. The phylogeny of purple bacteria: the gamma subdvision. System. Appl. Microbiol. 6: 25-33.

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Woese, C. R., E. Stackebrandt & W. Ludwig. 1985c. What are mycoplasmas: the relationship of tempo and mode in bacterial evolution. J. Mol. Evol. 21: 305-316.

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APPENDIX 1

Reagents

From Bresatec Ltd., South Aust.

α-^{35}S-dATP (concentration=12.5 mCi/ml, 8.8 nmol/ml; specific activity= 1470Ci/mmol)
α-^{33}P-dATP (concentration=10 mCi/ml, 8.5 nmol/ml; specific activity=1500Ci/mmol)
synthetic oligonucleotides

From Perkin Elmer Cetus, New Jersey, USA

Ampli-Taq® DNA Polymerase(5 U/µl)
GeneAmp® PCR Core Reagent Kit

From USB, Cleveland, Ohio

sterile mineral oil
dNTPs - 0.1M
Sequenase® Version 2 Kit
Sequenase® Version 2.0 DNA Polymerase (13 U/µl)

From Amresco, NSW, Aust.

Low-Range DNA Molecular Markers
phenol: chloroform: isoamyl alcohol (24:24:1) pH8.0
chloroform:isoamyl alcohol (24:1)

From Sigma, USA

nonidet P-40
EDTA-Na_2 (di-sodium ethylenediaminetetraacetate)
bromophenol blue
xylene cyanol FF
Proteinase K (12 U/mg solid)
Trizma base
Tris HCl
ammonium persulphate
ethidium bromide
sucrose
sodium hydroxide (anhydrous)
sodium chloride
DL-threonine
boric acid
RNAse A
sodium perchlorate

From Pharmacia LKB, NSW, Aust.

acrylamide
bis-acrylamide

From Boehringer Mannheim, W. Germany

lysozyme

From BDH, Vic. Aust.

sodium dodecylsulphate

From GIBCO BRL, Maryland UA

Ultrapure agarose, electrophoresis grade

From Univar, Ajax Chemicals, NSW, Aust.

acetic acid, reagent grade
ethanol, reagent grade

From Bio 101 Inc., La Jolla, CA USA

GENCLEAN II®Kit

From Eastman Kodak Co.

XOMAT-AR film

From Difco, Detroit, USA

nutrient broth
Columbian horse blood agar plates
brain-heart infusion broth

From Oxoid, England

Todd-Hewitt Broth

From FSE, NSW, Aust.

isoamyl alcohol

Stock Solutions

TAE - Tris-acetate buffer (Sambrook, 1989)
0.04M Tris-acetate, 0.001 EDTA

50x TAE:

Trizma-base	242 g
glacial acetic acid	57.1 ml
0.5M EDTA (pH8.0)	100 ml
ddH_2O to a final volume	1000 ml

TBE - Tris-borate buffer
0.89M Tris, 0.89M boric acid, 25mM EDTA

10x TBE (for agarose gels):

Trizma-base	108 g
boric acid	83 g
EDTA	9.3 g
ddH_2O to a final volume	1000 ml

Alternatively, 1M Tris, 0.83M boric acid, 10mM EDTA used for sequencing (Macrophor sequencing instructions)

10x TBE (for sequencing):

Trizma-base	121.1 g
boric acid	51.35 g
EDTA	3.72 g
ddH_2O to a final volume	1000 ml

40% acrylamide

Dissolve 95 g acrylamide
5 g NN'-methylenebisacrylamide
in 140 ml ddH_2O
Make up to 250 ml with ddH_2O. Store at 4°C in amber glass.

6% acrylamide

(Macrophor sequencing instructions)
Dissolve 42.0 g urea
14.5 ml 40% acrylamide stock
10.0 ml 10x TBE (sequencing)
in 40.5 ml ddH_2O
Make up to 99.2 ml with ddH_2O
Filter through 0.45µm filter. Store at 4°C in amber glass.

To each 100 ml add
- 0.8 ml ammonium persulphate (10%)
- 80 µl TEMED (N,N,N,N'-tetramethylenediamine).

ammonium persulphate (10%)

Dissolve 5 g ammonium persulphate in 40 ml ddH_2O. Make up to 50 ml with ddH_2O. Divide into 1 ml aliquots and store at -20°C.

ethidium bromide (10 mg/ml)

Dissolve in ddH_2O and store in lightproof tube at 4°C.

6x gel loading buffer

Dissolve 40 g sucrose in 80 ml dH_2O. Add 0.25 g bromophenol blue 6.25mM EDTA and 25 g SDS. Make up to 100 ml with ddH_2O.

or

bromophenol blue	0.25 g	(Sambrook, 1989)
xylene cyanol FF	0.25 g	
sucrose	40.0 g	
ddH_2O to a final volume	100 ml	

sodium dodecylsulphate (SDS) (10%)

Dissolve 10 g SDS in 85 ml ddH_2O. Make up to 100 ml with ddH_2o. Store at room temperature (22°C) for up to 4 months.

saline EDTA

NaCl	8.75 g
EDTA-Na_2	37.2 g

Dissolve in ddH_2O to 1 litre. Adjust to pH8.0 with anhydrous NaOH.

lysozyme (10 mg/ml)

Dissolve:

lysozyme	100 mg
ddH_2O	10 ml

Divide into 1 ml aliquots and store at -20°C.

proteinase K (10 mg/ml)

Dissolve:

proteinase K	100 mg
ddH_2O	10 ml

Divide into 1 ml aliquots and store at -20°C.
I suspect the use of saline EDTA to resuspend cells pre-lysis is a mistake with the use of Proteinase K, as it requires Ca^{2+} for optimal reactivity. In future use of this method, enzyme should be reconstituted with H_2O.

sodium perchlorate (70%)

Dissolve:

sodium perchlorate	70 g
ddH_2O to a final volume	100 ml

RNAse A

Dissolve 10 mg/ml RNAse A in 0.01M Tris-Cl (pH7.5), 0.015M NaCl. Heat at 100°C for 15 min. to destroy DNAse activity. Divide into 1 ml aliquots and store at -20°C (Sambrook et al., 1989).

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E. coli numbering (Gutell, 1993) is used throughout the text below to describe sites on the 16S rRNA molecule and gene sequence. Helices on the rRNA molecule are numbered according to Neefs et al., 1993.

16S rRNA sequence characters shared by *E. seriolicida* and *L. garvieae* are worthy of remark. These sites are all variable or highly variable across the prokaryotes (Neefs et al., 1993).

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i) position 723, where the consensus is U, the base shared by *E. seriolicida* and *L. garvieae* is A. This site occurs immediately before helix 26.

ii) positions 1120 and 1121 on helix 43. The consensus sequence over this doublet is G/CU (the gram positive cocci have G). The sequence presented here for *E. seriolicida* shows the doublet to be AC; the *L. garvieae* sequence an inconclusive doublet nC. The *E. seriolicida* sequence here maintains canonical base-pairing across the helix with complementary sequence alterations cited below in 4(i).

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2. The 16S rRNA molecule of the gram positive cocci has an extension to helix 6 occurring between positions 75 and 76. The length of the helix extension differs within and between these genera. For *E. seriolicida* and *L. garvieae* the helix is extended by 5 pairs of nucleotides; for other gram positive cocci the extension ranges between 4 and 9 pairs of nucleotides.

3. There are two sites within the sequence alignment at which *E. seriolicida* and *L. garvieae* concur in sequence but appear to differ from sequence conserved across related genera. At position 129, the enterococci, streptococci and most lactococci have a U; *L. cremoris* and *lactis* have G. *E. seriolicida* and *L. garvieae* have A. At position 192, the enterococci have G, the lactococci and *Streptococcus bovis* have U. *E. seriolicida* and *L. garvieae* have A.

4. There are 19 positions on the 16S molecule of *E. seriolicida* and *L. garvieae* with sequence identity that differ from all (or most) other sequences in the alignment. These sites, however, have little consensus in the alignment.

i) Of these, 12 involve complementary sequence changes across helices ie. higher order structural changes. These occur at 80-89, 154-167, 137-226, 139-224, 830-856, 1153-*1120* (mentioned in 1.ii above as a modified invariant site). Within the extension to helix 6 occurring between positions 75 and 76, *E. seriolicida* and *L. garvieae* have, uniquely, an A which pairs canonically within this extended helix to the U at the corresponding position. The other aligned sequences of gram positive cocci have either G, C or U.

ii) There is one site shared uniquely within the alignment by *E. seriolicida* and *L. garvieae* that transgresses canonical base-pairing within the 75-76 helix extension. *E. seriolicida* and *L. garvieae* have a U which pairs across the helix with another U. There is no sequence consensus at these sites in the alignment, and pairing is irregular.

iii) Seven other positions are shared by *E. seriolicida* and *L. garvieae* uniquely of the gram positive cocci, though no consensus at these sites occurs. Six are found within unpaired loops of the molecule (at positions 1260, 1274, 1275 between helices 45 and 46; at two positions in the extension between 183-184, and at one position between 193-194, before helix 10). One occurs within helix 10, at site 185 and the *E. seriolicida/L. garvieae* sequence maintains canonical base-pairing.

APPENDIX 3

Precedents for Flowery Language in Bacterial Systematics.

Certain accusations of "flowery" language have been levelled at passages of this thesis by supervisors who will remain nameless. I would maintain, on the contrary, that semantics herein are actually both precisely informative and unambiguous, though perhaps abjuring scientific jargon. The quotations cited hereunder testify to a solid tradition of descriptive licence in bacterial taxonomy, some delightful, some circumventing meaning altogether (eg. Wheelis, Kandler & Woese, 1992).

George Fox et al., 1980: The Phylogeny of the Prokaryotes. Science 209, 457-463.

* "What had been a dry, esoteric, and uncertain discipline.....is becoming a field fresh with the excitement of the experimental harvest."

Carl Woese, 1992. Prokaryotic systematics: the evolution of a science. In: The Prokaryotes, 2nd edition.

* "...the phenotypic quagmire of ill-defined.....characters.."

* "...a convenient and powerful device by which prejudices could be laundered into scientifically respectable pronouncements."

* "...construction of phylogenetic trees has become an arcane art."

* "...speculation concerning bacterial evolution became solely an extracurricular activity, best done over a glass of port..."

* " ...the seductive symmetry of this simplistic dichotomy..."

* "...on this latter cosmopolitan branch of the tree.."

* " ... became ostensible grounds for vitiating Orla-Jensen's claim...."

* ".... who evinced little cognisance of evolution."

* "This taxonomic "double standard" undoubtedly contributes to our mistaken impression that microbial evolution has a far more protean quality than metazoan..."

Carl Woese et al., 1985. A phylogenetic definition of the major eubacterial taxa. System. Appl. Microbiol. 6: 143-151.

* "... and higher taxa, if defined at all, were no more than expressions of prejudice..."

Gary Olsen & Carl Woese, 1993. Ribosomal RNA: a key to phylogeny. FASEB J. 7: 113-123.

* " from the vantage point of molecular data, he [the evolutionist] now gazes over the Cambrian "wall" that has obstructed his temporal perspective..."

* "colour has been added to his monochromic, morphocentric view of the evolutionary process..."

* " ... static and relatively superficial palaeontological link between biology and geology becomes engulfed by the far more interesting long-term interplay between the evolution of the physical planet and that of the organisms inhabiting it."

* "Here was a science that, devoid of an evolutionary framework... had developed in a stunted way."

* "... a vast and rich mine of evolutionary information.."

* "With this publication, the handwriting was on the wall ...."

* " ..... the genotypic variety is continually roiling, producing a profusion of change ..."

* ".... for reasons we will not discuss here, microbiologists did not harken to the molecular message..."

* "... an extraordinary amount of energy (and bile) has gone into arguments....."

* "... another classical taxonomic decideratum, gliding motility..." - *not just flowery but a pun in nouveau Latin!!!* (it's actually just wrong).

* "the crenarchaeotes are phenotypically monotonous..."

* "mitochondria are restricted to organisms found in the crown of the eukaryotic tree..."

Edward Wilson, 1985. Time to revive systematics. Science 230: 13.

* "the resulting genome is richer in content than a Caravaggio painting, a Bach fugue...."

* "the community of systematists is sadly inadequate to the immense task..."

Olsen et al. 1994. The winds of (evolutionary) change: breathing new life into microbiology. J. Bacteriol. 176: 1-6.

* ".... that evolution....must be the conceptual heart of biology...."

* "the most profound symptom of microbiology's unfortunate condition was its reliance on the prokaryote-eukaryote dichotomy as a phylogenetic crutch...."

* "with repetition (as catechism) the dichotomy served only to make microbiologists easily accept their near total ignorance.."

Krieg. 1988. Bacterial classification: an overview. Can. J. Microbiol. 34: 536-540.

* "Linnaeus believed that species were more or less made in heaven ..."

Mark Wheelis et al. 1992. On the nature of global classification. Proc. Natl. Acad. Sci. USA 89: 2930-2934.

* "...to define the overarching concept of biology, pedagogically as well as experimentally."

* "Linnaean classification represents a scientific codification of this organising proclivity."

* "global systematics should be as complete an encapsulation of the evolutionary paradigm as possible...."

Carl Woese. 1987. Macroevolution in the microscopic world. In: Molecules or Morphology: Conflict or Compromise?. pp. 177-202. C. Patterson (ed.). Cambridge University Press, Cambridge U.K.

* "...but even some of these, it turns out, were phylogenetic monstrosities."

* "..for the subject is too ill-defined for it to be anything other than an exercise in unbridled speculation."

Woese et al. 1990. Towards a natural system of organisms. Proc. Natl. Acad. Sci. USA 87: 4576-4579.

* "..is still largely steeped in the ancient notion that...."

* "... divest ourselves of deeply ingrained and cherished assumptions..."

Carl Woese, 1987. Bacterial evolution. Microbiol. Rev. 51: 221-271.

* " ...the Linnaean classification system, stultifying in its liturgy..."

* "...captured the spirit of the times in this piquant proscription..."

* "...the two small clouds on this horizon ..... are thermophiles noted for their unusual lipids."

* "macroevolution in mycoplasmas is chronic."

* "its mutation rate would necessarily return to normal, but the organism would bear the scars of its tumultuous history."

* "a molecular chronometer will show structural idiosyncrasies in the latter case, the "scars" of the lineage's bout of rapid evolution."

* "bacterial evolution is no simple extrapolation of metazoan evolution - more of the same and lacking fossils...."

* "the progenote is today the end of an evolutionary trail that starts with fact, progresses through inference, and fades into fancy."

APPENDIX 1

Reagents

From Bresatec Ltd., South Aust.

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α-^{33}P-dATP (concentration=10 mCi/ml, 8.5 nmol/ml; specific activity=1500Ci/mmol)
synthetic oligonucleotides

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GeneAmp® PCR Core Reagent Kit

From USB, Cleveland, Ohio

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dNTPs - 0.1M
Sequenase® Version 2 Kit
Sequenase® Version 2.0 DNA Polymerase (13 U/µl)

From Amresco, NSW, Aust.

Low-Range DNA Molecular Markers
phenol: chloroform: isoamyl alcohol (24:24:1) pH8.0
chloroform:isoamyl alcohol (24:1)

From Sigma, USA

nonidet P-40
EDTA-Na_2 (di-sodium ethylenediaminetetraacetate)
bromophenol blue
xylene cyanol FF
Proteinase K (12 U/mg solid)
Trizma base
Tris HCl
ammonium persulphate
ethidium bromide
sucrose
sodium hydroxide (anhydrous)
sodium chloride
DL-threonine
boric acid
RNAse A
sodium perchlorate

From Pharmacia LKB, NSW, Aust.

acrylamide
bis-acrylamide

From Boehringer Mannheim, W. Germany

lysozyme

From BDH, Vic. Aust.

sodium dodecylsulphate

From GIBCO BRL, Maryland UA

Ultrapure agarose, electrophoresis grade

From Univar, Ajax Chemicals, NSW, Aust.

acetic acid, reagent grade
ethanol, reagent grade

From Bio 101 Inc., La Jolla, CA USA

GENCLEAN II® Kit

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0.89M Tris, 0.89M boric acid, 25mM EDTA

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Alternatively, 1M Tris, 0.83M boric acid, 10mM EDTA used for sequencing (Macrophor sequencing instructions)

10x TBE (for sequencing):

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40% acrylamide

Dissolve 95 g acrylamide
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in 140 ml ddH_2O
Make up to 250 ml with ddH_2O. Store at 4°C in amber glass.

6% acrylamide

(Macrophor sequencing instructions)
Dissolve 42.0 g urea
14.5 ml 40% acrylamide stock
10.0 ml 10x TBE (sequencing)
in 40.5 ml ddH_2O
Make up to 99.2 ml with ddH_2O
Filter through 0.45µm filter. Store at 4°C in amber glass.

To each 100 ml add
- 0.8 ml ammonium persulphate (10%)
- 80 µl TEMED (N,N,N,N'-tetramethylenediamine).

ammonium persulphate (10%)

Dissolve 5 g ammonium persulphate in 40 ml ddH_2O. Make up to 50 ml with ddH_2O. Divide into 1 ml aliquots and store at -20°C.

ethidium bromide (10 mg/ml)

Dissolve in ddH_2O and store in lightproof tube at 4°C.

6x gel loading buffer

Dissolve 40 g sucrose in 80 ml dH_2O. Add 0.25 g bromophenol blue 6.25mM EDTA and 25 g SDS. Make up to 100 ml with ddH_2O.

or

bromophenol blue	0.25 g	(Sambrook, 1989)
xylene cyanol FF	0.25 g	
sucrose	40.0 g	
ddH_2O to a final volume	100 ml	

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Dissolve 10 g SDS in 85 ml ddH_2O. Make up to 100 ml with ddH_2o. Store at room temperature (22°C) for up to 4 months.

saline EDTA

NaCl	8.75 g
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Dissolve in ddH_2O to 1 litre. Adjust to pH8.0 with anhydrous NaOH.

lysozyme (10 mg/ml)

Dissolve:

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Certain accusations of "flowery" language have been levelled at passages of this thesis by supervisors who will remain nameless. I would maintain, on the contrary, that semantics herein are actually both precisely informative and unambiguous, though perhaps abjuring scientific jargon. The quotations cited hereunder testify to a solid tradition of descriptive licence in bacterial taxonomy, some delightful, some circumventing meaning altogether (eg. Wheelis, Kandler & Woese, 1992).

George Fox et al., 1980: The Phylogeny of the Prokaryotes. Science 209, 457-463.

* "What had been a dry, esoteric, and uncertain discipline.....is becoming a field fresh with the excitement of the experimental harvest."

Carl Woese, 1992. Prokaryotic systematics: the evolution of a science. In: The Prokaryotes, 2nd edition.

* "...the phenotypic quagmire of ill-defined.....characters.."

* "...a convenient and powerful device by which prejudices could be laundered into scientifically respectable pronouncements."

* "...construction of phylogenetic trees has become an arcane art."

* "...speculation concerning bacterial evolution became solely an extracurricular activity, best done over a glass of port..."

* " ...the seductive symmetry of this simplistic dichotomy..."

* "...on this latter cosmopolitan branch of the tree.."

* " ... became ostensible grounds for vitiating Orla-Jensen's claim...."

* ".... who evinced little cognisance of evolution."

* "This taxonomic "double standard" undoubtedly contributes to our mistaken impression that microbial evolution has a far more protean quality than metazoan..."

Carl Woese et al., 1985. A phylogenetic definition of the major eubacterial taxa. System. Appl. Microbiol. 6: 143-151.

* "... and higher taxa, if defined at all, were no more than expressions of prejudice..."

Gary Olsen & Carl Woese, 1993. Ribosomal RNA: a key to phylogeny. FASEB J. 7: 113-123.

* " from the vantage point of molecular data, he [the evolutionist] now gazes over the Cambrian "wall" that has obstructed his temporal perspective..."

* "colour has been added to his monochromic, morphocentric view of the evolutionary process..."

* " ... static and relatively superficial palaeontological link between biology and geology becomes engulfed by the far more interesting long-term interplay between the evolution of the physical planet and that of the organisms inhabiting it."

* "Here was a science that, devoid of an evolutionary framework... had developed in a stunted way."

* "... a vast and rich mine of evolutionary information.."

* "With this publication, the handwriting was on the wall ...."

* " ..... the genotypic variety is continually roiling, producing a profusion of change ..."

* ".... for reasons we will not discuss here, microbiologists did not harken to the molecular message..."

* "... an extraordinary amount of energy (and bile) has gone into arguments....."

* "... another classical taxonomic decideratum, gliding motility..." - *not just flowery but a pun in nouveau (incorrect)Latin!!*

* "the crenarchaeotes are phenotypically monotonous..."

* "mitochondria are restricted to organisms found in the crown of the eukaryotic tree..."

Edward Wilson, 1985. Time to revive systematics. Science 230: 13.

* "the resulting genome is richer in content than a Caravaggio painting, a Bach fugue...."

* "the community of systematists is sadly inadequate to the immense task..."

Olsen et al. 1994. The winds of (evolutionary) change: breathing new life into microbiology. J. Bacteriol. 176: 1-6.

* ".... that evolution....must be the conceptual heart of biology...."

* "the most profound symptom of microbiology's unfortunate condition was its reliance on the prokaryote-eukaryote dichotomy as a phylogenetic crutch...."

* "with repetition (as catechism) the dichotomy served only to make microbiologists easily accept their near total ignorance.."

Krieg. 1988. Bacterial classification: an overview. Can. J. Microbiol. 34: 536-540.

* "Linnaeus believed that species were more or less made in heaven ..."

Mark Wheelis et al. 1992. On the nature of global classification. Proc. Natl. Acad. Sci. USA 89: 2930-2934.

* "...to define the overarching concept of biology, pedagogically as well as experimentally."

* "Linnaean classification represents a scientific codification of this organising proclivity."

* "global systematics should be as complete an encapsulation of the evolutionary paradigm as possible...."

Carl Woese. 1987. Macroevolution in the microscopic world. In: Molecules or Morphology: Conflict or Compromise?. pp. 177-202. C. Patterson (ed.). Cambridge University Press, Cambridge U.K.

* "...but even some of these, it turns out, were phylogenetic monstrosities."

* "..for the subject is too ill-defined for it to be anything other than an exercise in unbridled speculation."

Woese et al. 1990. Towards a natural system of organisms. Proc. Natl. Acad. Sci. USA 87: 4576-4579.

* "..is still largely steeped in the ancient notion that...."

* "... divest ourselves of deeply ingrained and cherished assumptions..."

Carl Woese, 1987. Bacterial evolution. Microbiol. Rev. 51: 221-271.

* " ...the Linnaean classification system, stultifying in its liturgy..."

* "...captured the spirit of the times in this piquant proscription..."

* "...the two small clouds on this horizon ..... are thermophiles noted for their unusual lipids."

* "macroevolution in mycoplasmas is chronic."

* "its mutation rate would necessarily return to normal, but the organism would bear the scars of its tumultuous history."

* "a molecular chronometer will show structural idiosyncrasies in the latter case, the "scars" of the lineage's bout of rapid evolution."

* "bacterial evolution is no simple extrapolation of metazoan evolution - more of the same and lacking fossils...."

* "the progenote is today the end of an evolutionary trail that starts with fact, progresses through inference, and fades into fancy."